Characterization of Imipenem-resistant *Pseudomonas aeruginosa* in Hong Kong

YIP, Yuen Fong

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Abstract

A total of 140 single patient isolates of imipenem-resistant *Pseudomonas aeruginosa* from the New Territories East Cluster hospitals (NTEC), Hong Kong, collected during 2001 - 2005 were tested for their susceptibility to 12 β-lactams and seven other antibiotics. Mechanisms of imipenem-resistance including production of β-lactamases, changes in membrane permeability, efflux and presence of integrons were investigated. More than 50% of these isolates were resistant to ten and up to 19 antibiotics. Only one isolate each produced an OXA-3 and an OXA-10 β-lactamase. Twenty isolates were shown by an imipenem-EDTA disk method to produce metallo-beta-lactamase (MBL) but MBL genes including bla_{IMP}, bla_{VIM} and bla_{SIM} could not detected in the isolates after amplification using primers specific for these genes. Crude β-lactamase extracts from these isolates were unable to hydrolyze imipenem.

Reduced expression of the *oprD* gene was detected in nine out of the 28 isolates that were selected for further study of the mechanisms of imipenem-resistance. Amplification followed by DNA sequence determination of the *mexT* gene, the negative regulator of *oprD*, showed that mutations were present in the *mexT* gene in 19 isolates. These mutations led to the *mexT* gene to become functional and so had down-regulated the expression of *oprD*.

A class 1 integron was detected in 94 of the 140 isolates studied. The bla_{OXA-3}, bla_{OXA-10}, *aadA1*, *aadA2* and *aadA6* genes were present in gene cassettes of the integrons. All 20 isolates shown to produce a MBL by a bioassay method harbored a class 1 integron but the 3' conserved region was not detected in 16 of these isolates.

Imipenem-resistant *P. aeruginosa* isolates in the NTEC hospitals were usually multiply drug-resistant. Production of carbapenemase was not likely to contribute to imipenem-resistance. Loss of OprD protein, and overexpression of an efflux pump system were probably mechanisms of imipenem-resistance in our isolates.

摘要

針對由香港新界東聯網醫院在 2001 至 2005 年間所分離出的 140 株單一病人抗亞胺培南(imipenem)綠膿桿菌，進行了 12 種β-內酰胺類和其他 7 種抗生素敏感性測試。此論文研究的抗亞胺培南機制包括β-內酰胺酶的製造、膜通透性的改變、基因調節 integrons 的出現。研究結果顯示，多於 50%菌種對十至十九種抗生素呈抗藥性反應，其中只有 OXA-3 β-內酰胺酶和 OXA-10 β-內酰胺酶分別於兩個獨立菌種中發現。在混合亞胺培南藥片和乙二胺四乙酸(EDTA)的測試方法中，共有 20 株菌種能生產出金屬內酰胺酶(MBL)，但利用聚合酵素連鎖反應 (PCR) 的方法，金屬內酰胺酶基因包括 bla_{IMP}, bla_{VIM} 和 bla_{SIM} 皆未能在菌種中顯示。研究指出未經提煉的β-內酰胺酶不能水解亞胺培南。

28 株被揀選作進一步研究抗亞胺培南機制中，有 9 株菌種發表 *oprD* 外膜蛋白基因的能力減低。*mexT* (*OprD* 的陰性調節器) 的核酸排序 (DNA sequencing)中發現，19 株菌種有基因突變。這些基因突變令 *mexT* 基因變得活躍，從而減低發表 *oprD*。但在報告中，我們也提出其他影響調控 *oprD* 的因素。

在 140 株菌種中，發現第一類 integron 的有 94 株。在這些 integron 的基因盒(gene cassette)裏，*bla*$_{OXA-3}$, *bla*$_{OXA-10}$, *aadA1, aadA2* 和 *aadA6* 基因均有出現。製造金屬內酰胺酶的 20 株菌種都懷有第一類 integron，但 3'保存部份的第一類 integron 基因盒中，未能在其他 16 株菌種中發現。

在新界東聯網醫院中，抗亞胺培南綠膿桿菌常是抗多種藥物性的。環烯類胺(carbapamases)的生產不很可能是這些菌種引致對亞胺培南抵藥的因素，而沒有因素被確認是唯一能抵藥機制。OprD 蛋白的流失、過度生產的 AmpC β-內酰胺胺和過度發表的藥物排泵系統(efflux pump system)更有可能在這些菌種相互影響來抵抗亞胺培南

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Chapter 1-Introduction

1 *Pseudomonas aeruginosa*

1.1 Microbiology

Pseudomonas aeruginosa is the pseudomonad species that is most frequently recovered from clinical specimens. It is an oxidase-positive, non-fastidious Gram-negative aerobe that derives its energy from oxidation rather than fermentation of glucose (Koneman *et al.*, 1997) (Figure 1.1).

1.1.1 Morphology

Most *P. aeruginosa* strains form characteristic blue-green colonies on nutrient agar due to the production of pyocyanin and fluorescein. These colonies are smooth and flat and most of them have a distinguishable fruity odor due to formation of *o*-aminoacetophenone from tryptophan. *P. aeruginosa* grows well at 25°C to 37°C and is able to grow, albeit slower, at a lower or higher temperature; its ability to grow at 42°C distinguishes it from many other *Pseudomonas* species (Murray *et al.*, 2005).

The cells of *P. aeruginosa* are rod-shaped, measuring 0.5 - 0.8 μm by 1.5 - 3.0 μm. Most of the strains are motile by one or more polar flagellum. Clinical isolates usually have pili, which are antiphagocytic and aid in attachment to host cells for colonization.

1.1.2 Identification

P. aeruginosa is identified by its inability to ferment lactose, a positive oxidase reaction, its ability to grow at 42°C and various other biochemical reactions (Murray *et al.*, 2005).

1.1.3 Pathogenesis and virulence

P. aeruginosa produces many virulence factors. These include heat-stable hemolysin, phospholipase C, endotoxin, pigments (pyocyanin and fluorescein), proteases (elastase and alkaline protease), toxin A and exoenzyme S (Mandell *et al.*, 2005; Murray *et al.*, 2005).

1.1.4 Host defenses

Phagocytosis is an important host defense mechanism and together with opsonizing antibody, protecting the host against *P. aeruginosa* infections. Patients unable to produce antibodies due to various underlying diseases or immunosuppressive therapies usually have more serious *P. aeruginosa* infections (Mandell *et al.*, 2005).

1.1.5 Epidemiology

P. aeruginosa commonly inhabits soil, water, and vegetation. It is a normal commensal of the skin, throat and stool of healthy persons. *P. aeruginosa* more frequently causes hospital-acquired infections than community-acquired infections. It is also resistant to some commonly-used disinfectants and is therefore a contaminant of disinfectants. It has also been isolated from respiratory equipment, food, sinks, taps and mops in hospitals. It is mainly acquired during invasive procedures or transmitted from patients to patients and from health care personnel to patients (Mandell *et al.*, 2005).

1.1.6 Clinical manifestations

P. aeruginosa is the third most common cause of nosocomial infections after *Escherichia coli* and *Staphylococcus aureus* (Livermore and Pearson, 1996). Recent surveys have shown that *P. aeruginosa* is responsible for 10% - >20% of all nosocomial infections in the United States (NNIS, 1996). It can cause life-threatening infections such as bacteremia, pneumonia, etc in patients with altered host defenses, cystic fibrosis, wounds and burns or on chemotherapy (Mandell *et al.*, 2005). Fatality rate can be as high as 50% among patients with these risk factors (Mandell *et al.*, 2005).

1.1.7 Treatment

Although many antibiotics have modest antipseudomonal activity in vitro, only a minority of them show useful activity at therapeutically attainable concentrations. Carbenicillin is the first commercially available penicillin to show activity against *P. aeruginosa*. The other antipseudomonal penicillins include ticarcillin, azlocillin and piperacillin. Those cephalosporins that are active against *P. aeruginosa* include cefoperazone, cefsulodin, ceftazidime, cefpirome and cefepime. The monobactam aztreonam has also been reported to have antipseudomonal activity, however, the carbapenems such as imipenem and meropenem are the most active β-lactams against this organism (Cunha and Ristuccia, 1984). Newer carbapenems that have recently been developed include ertapanem and doripenem (Zhanel *et al.*, 2005).

The aminoglycosides are also active against *P. aeruginosa*. Despite of their nephrotoxicity and ototoxicity (Cunha and Ristuccia, 1984), life-threatening *P.*

aeruginosa infections are often treated with a combination of an aminoglycoside and a β-lactam agent that has excellent synergistic activities (Cunha and Ristuccia, 1984).

Fluoroquinolones have also been used to treat pseudomonal infections. Ciprofloxacin is the most widely used fluoroquinolone in recent years. There is often an improvement in lung function with ciprofloxacin therapy in cystic fibrosis patients (Raeburn *et al.*, 1987; Wolfson and Hooper, 1989).

2 β-Lactams

The β-lactams are probably the most varied and widely used of all antimicrobial agents due to their low toxicity and high availability (Lorian, 1995). Benzylpenicillin is the first β-lactam that was discovered in 1928 but was put into clinical use in 1940. There are numerous natural and synthetic compounds described based upon the active structure of penicillin, the β-lactam ring (Figure 1.2).

The penicillins and cephalosporins, which are based on 6-aminopenicillanic acid and 7-aminocephalosporanic acid, respectively, are the two classical β-lactam families. Penicillins such as benzylpenicillin are mainly active against Gram-positive bacteria while others such as ampicillin and carbenicillin are active against Gram-negative bacteria (Brumfitt *et al.*, 1967; Lorian, 1995). Cephalosporins can be classified into generations according to their antibacterial spectrum. The first generation cephalosporins, such as cephalothin and cephradine, are mainly active against Gram-positive organisms. The second generation cephalosporins are more active against Gram-negative bacteria than the first generation cephalosporins but their activity against the Gram-positives decreases (Wise, 1992). The third generation cephalosporins are very active against most Gram-negative organisms but are much less active against Gram-positive organisms than the first or second generation cephalosporins. Most cephalosporins do not have good

antipseudomonal activity except the third generation cephalosporins, including cefpirome, cefoperazone, cefsulodin and ceftazidime, and the fourth generation cefepime of the so-called antipseudomonal cephalosporins (Williams and Moosdeen, 1987; Duval *et al.*, 1993; Thornsberry *et al.*, 1993).

In addition, various nonclassical β-lactams have been developed, including monobactams and carbapenems. Monobactams have been used extensively in the treatment of a variety of infections caused by Gram-negative pathogens, including *P. aeruginosa*. Its pharmacologic profile can be attributed to its unique chemical property. Monobactams have a single β-lactam ring structure, which differs substantially from those of the bicyclic-lactams, such as the penicillins and cephalosporins. Aztreonam is the only clinically used monobactam (Sykes *et al.*, 1981; Sykes and Bonner, 1985).

Carbapenems are β-lactams with the broadest spectrum of activity. Also, they are very stable to most β-lactamases, more rapidly bactericidal than most other penicillins or cephalosporins and have been used to treat infections caused by most bacterial pathogens except mycobacteria, cell wall-deficient organisms, and a few infrequent non-fermenters and aeromonads. They are not hydrolyzed by extended-spectrum β-lactamases (ESBLs) and are active against hyperproducers of AmpC enzymes (Hashizume *et al.*, 1984; Livermore and Woodford, 2000). Carbapenems differ from conventional penicillins in having no sulfur atom in their 5-membered ring, instead, they have a double bond between carbons 2 and 3 (Figure 1.3). The first carbapenem to be used clinically is imipenem in the 1980s (Walsh *et al.*, 2005). Other carbapenems include meropenem, ertapenem and doripenem (Zhanel *et al.*, 2007), with ertapenem having the most limited spectrum of activity because it is inactive against *P. aeruginosa* and *Enterococcus* sp (Wexler, 2004).

β-Lactamase inhibitors are β-lactams but they do not have any antimicrobial properties. They are usually used in combination with those β-lactams that are sensitive to β-lactamases. Clinically-used β-lactamase inhibitors include clavulanic acid that is combined with amoxicillin and ticarcillin and the penicillanic acid sulfone sulbactam and tazobactam, the former combined with ampicillin and the latter with piperacillin (Rolinson, 1991).

2.1 Mode of action of β-lactams

β-Lactams are bactericidal since they inhibit synthesis of peptidoglycan (Tipper and Strominger, 1965; Waxman and Strominger, 1983), the major polymer of the bacterial cell wall. Peptidoglycan plays an essential role in bacteria by maintaining the cell shape and protects against osmotic forces. In Gram-positive organisms, the peptidoglycan forms a thick layer external to the cytoplasmic membrane while in Gram-negative bacteria and mycobacteria, it forms a thin layer between the outer and cytoplasmic membrane (Nikaido, 1994).

β-Lactams have an affinity for penicillin binding proteins (PBPs), which are the enzymes responsible for cell wall synthesis and thus are targets of β-lactams. Binding of β-lactams to a cell causes it to swell and finally to burst (Spratt 1975; Frere and Joris, 1985).

2.2 β-Lactam-resistance

β-Lactams are the most widely used antimicrobial agents clinically. However, emerging β-lactam-resistant strains are found in many parts of the world. β-Lactam-

resistance results in treatment failure and therefore is a global life-threatening health problem.

2.3 Resistance mechanisms

Mechanisms of β-lactam-resistance can be either acquired or intrinsic. The mechanisms primarily include alteration of bacterial target, reduction of the uptake of antibiotics into bacterial cells, production of enzymes that degrade or modify the antibiotics, and presence of efflux pump systems.

2.3.1 Changes in PBPs

An important mechanism of resistance to β-lactams is the alteration of PBPs. The resistant strains produce another PBP that has decreased affinity to the antibiotics in order to take up the function of the original protein and to escape the action of the antibiotics. Alterations in PBPs are an important mechanism of β-lactam-resistance of Gram-positive bacteria, such as *S. aureus*, *Streptococcus pneumoniae* and Gram-negative bacteria, such as *Neisseria gonorrhoeae* and *Pseudomonas* sp (Godfrey *et al.*, 1981; Spratt 1988; Spratt, 1994).

The change in PBPs may be due to acquisition of a single foreign gene, such as the *mecA* gene that codes for a variant of PBP 2, designated PBP 2a, in *S. aureus*. Since PBP 2a has low affinity for all β-lactams, production of PBP 2a leads to the development of resistance to all β-lactams in *S. aureus* (Ubukata *et al.*, 1989). In contrast, *S. pneumoniae* takes up segments of genes that code for PBPs released from dead β-lactam-resistant bacteria and then inserts them into its own PBP genes to form "mosaic genes". These "mosaic genes" code for PBPs that have very low affinity to β-lactams thus

resulting in β-lactam-resistance. *S. pneumoniae* strains that are resistant to high concentrations of penicillin have changes in four PBPs while those resistant to low concentrations have changes in only three (Jabes *et al.*, 1989).

2.3.2 Impermeability

Hydrophilic antimicrobial agents enter into Gram-negative bacteria by diffusion through channels formed by "porin" proteins that are present in the outer membrane (Nikaido, 1985; Nikaido and Vaara 1985). Alterations in porin gene expression that lead to fewer porins or porins of a different size or structure decrease the amount of an antibiotic that can enter into the bacterial cell. Since different classes of antibiotics may use the same porin to enter the cell, alterations in outer membrane porins that affect permeability to one drug may affect permeability to a number of unrelated antimicrobial agents. Loss of porins is one of the mechanisms of β-lactam-resistance in Gram-negative organisms, including *E. coli*, *Proteus* sp, *P. aeruginosa, Acinetobacter baumannii, Serratia marcescens*, and *Klebsiella pneumoniae* (Raimondi *et al.*, 1995; Clarke *et al.*, 2003; Quale *et al.*, 2003). In *E. coli*, mutations in outer membrane protein encoding genes *omp*F and *omp*C lead to structural alterations in the protein and are associated with β-lactam resistance (Yoshimura and Nikaido, 1985). These mechanisms render bacteria to become resistant to high concentrations of antibiotics (Martinez-Martinez *et al.*, 1996).

2.3.3 β-Lactamases

β-Lactamases are the major defence of Gram-negative bacteria against β-lactams. They disrupt the amide bond of the β-lactam ring thus rendering the antimicrobial inactive (Majiduddin *et al.*, 2002; Helfand *et al.*, 2003). No sooner has one new β-lactam

been developed and put into clinical use one or more bacterial species start to produce a β-lactamase that can hydrolyze it (Medeiros, 1997). As a result there are as many β-lactamases as there as new β-lactams that have been developed to specifically resist hydrolysis by β-lactamases. Thus, there is a β-lactamase that can hydrolyze each class of β-lactams.

Shah and colleagues reported in 2004 that there are >340 known β-lactamases (Shah *et al.*, 2004) and there are certainly many more β-lactamases reported since then. These β-lactamases are encoded by genes that can be borne on a transposon or plasmid or on the chromosome. Chromosomal β-lactamases show some sequence homology with PBPs and are thus thought to have evolved from them (Ghuysen, 1991). The rapid spread of β-lactamases among clinical isolates is due to encoding genes being borne on a plasmid or transposon (Bradford, 2001). The TEM-1 enzyme, the first plasmid-mediated β-lactamase discovered, was reported in the 1960s' in Greece (Medeiros, 1984). Since then, it and its variants develop and spread rapidly worldwide and are the most common plasmid-mediated β-lactamases produced by bacteria within the family *Enterobacteriaceae* (Philippon *et al.*, 1994).

β-Lactamases can be classified into four molecular classes based on sequence (classes A to D) or into 11 functional groups based on substrate profiles (the substrates they can hydrolyse) and inhibitor profiles (the inhibitors that can inhibit their activities) (Ambler *et al.*, 1991; Bush *et al.*, 1995).

Class A β-lactamases include both chromosomal and plasmid-mediated enzymes and are inhibited by clavulanic acid. Class B β-lactamases, also known as metallo-enzymes (MBLs), require zinc for activity, are resistant to classical β-lactamase inhibitors but susceptible to EDTA. Class C enzymes, also known as AmpC enzymes, are

chromosomal cephalosporinases that are resistant to inhibition by clavulanic acid and are typically produced by Enterobacteria. AmpC β-lactamases are mainly found in *K. pneumoniae* and *E. coli* worldwide (Philippon *et al.*, 2002). Class D enzymes are also known as oxacillinases since they hydrolyze oxacillin more effectively than benzylpenicillin. Classes A, C and D β-lactamases have serine as their active site (Bush *et al.*, 1995). β-Lactamases of classes A and D are the most common. Class A enzymes such as TEM-type, SHV-type and CTX-M-type are commonly produced by Gram-negative bacteria such as *E. coli* while class D enzymes such as OXA-type are frequently produced by *P. aeruginosa* (Medeiros *et al.*, 1984).

2.3.3.1 Extended spectrum β-lactamases

Extended spectrum β-lactamase are co-called since they can hydrolyze the extended-spectrum β-lactams that include the third generation cephalosporins and monobactams. They are mainly derivatives of TEM-1 and SHV-1 enzymes due to presence of point mutations in the encoding genes, which are often located on transferable plasmids (Jacoby and Medeiros, 1991; Bush *et al.*, 1995) (http://www.lahey.org/studies).

More than 130 TEM-type ESBLs have been identified. TEM-10, TEM-12, and TEM-26 are among the most common in North and South America (Paterson *et al.*, 2003). Another common ESBLs are the SHV-type enzymes. More than 50 SHV-type enzymes have been reported and are mainly detected in resistant clinical isolates in Europe and America (Yuan *et al.*, 1998; Paterson *et al.*, 2003). The SHV-5 and SHV-12 are the most common members among the SHV family (Paterson *et al.*, 2003). Both TEM-type and SHV-type ESBLs are most commonly found in *K. pneumoniae* and *E. coli* but they are

also found in many other *Enterobacteriaceae*, such as *Proteus* sp and *Providencia* sp (Livermore 1995; Rasheed *et al.*, 1997; Bonnet *et al.*, 1999; Marchandin *et al.*, 1999). ESBLs, such as TEM-42 and SHV-2a β-lactamases, have also been found in *P. aeruginosa* isolates (Mugnier *et al.*, 1996; Naas *et al.*, 1999).

The most common group of ESBLs not belonging to the TEM or SHV families was termed CTX-M, which has a greater activity to hydrolyze cefotaxime. More than 40 CTX-M enzymes have been described. They have been found in strains of *Salmonella enterica* serovar Typhimurium and *E. coli*, but have also been described in other species of *Enterobacteriaceae* (Bonnet, 2004). Strains with CTX-M-type β-lactamases have been isolated from many parts of the world and have often been associated with focal outbreaks in Eastern Europe (Gazouli *et al.*, 1998; Gniadkowski *et al.*, 1998), South America (Bradford *et al.*, 1998), and Japan (Ma *et al.*, 1998).

The OXA-type enzymes are another growing family of ESBLs. Unlike most of the other ESBLs, which have been found in *E.* coli, *K. pneumoniae*, and other *Enterobacteriaceae*, the OXA-type ESBLs are mainly produced by *P. aeruginosa* (Danel *et al.*, 1997; Hall *et al.*, 1993; Poirel *et al.*, 2001a). Currently, more than 120 different variants of OXA-type ESBL have been identified (Walther-Rasmussen and Hoiby, 2006).

2.3.3.2 Carbapenemases

Carbapenemases are β-lactamases of classes A, B or D that significantly hydrolyze imipenem and / or meropenem and are one of the mechanisms of carbapenem-resistance. The number of carbapenamases has increased over the last few years and at least seven types have been reported (Walsh *et al.*, 2005). These β-lactamases can be either chromosomally- or plasmid-mediated. Bacterial species that produce chromosomal

carbapenemases are usually environmental organisms such as *Flavobacterium* (Bellais *et al.*, 1999) and *Chryseobacterium* sp (Bellais *et al.*, 2000), *Aeromonas hydrophila* (Massidda *et al.*, 1991), *Legionella gormanii* (Boschi *et al.*, 2000) and *Janthinobacterium lividum* (Rossolini *et al.*, 2001).

A few class A carbapenamases have been reported in rare enterobacterial species. These carbapenamases may not mediate high-level imipenem-resistance or be inhibited by clavulanic acid (Poirel and Nordmann, 2002). NMC-A is the first class A carbapenemase identified in France in 1990 in an *Enterobacter cloacae* clinical isolate (Nordmann *et al.*, 1993). Other class A carbapenemases identified so far include SME-1 to SME-3 (Yang *et al.*, 1990), IMI-1 (Rasmussen *et al.*, 1996), KPC-1 (Yigit *et al.*, 2001) and GES-2 (Poirel *et al.*, 2001b).

The class B metallo-β-lactamases (MBL) are the most common carbapenamases. The encoding genes are transferable and have been detected in a variety of Gram-negative organisms, especially *P. aeruginosa* and *A. baumanni* (Walsh *et al.*, 2005). In Korea, *P. aeruginosa* producing MBLs accounts for nearly 20% of all nosocomial isolates (Lee *et al.*, 2003). MBLs have a very broad substrate profile that includes expanded-spectrum cephalosporins (such as cefotaxime, ceftazidime, and cefepime) and carbapenems but not the monobactams (Haruta *et al.*, 2000).

The IMP-type carbapenemases is the most common group of MBLs. IMP-1, the first carbapenemase, was identified in a *S. marcescens* strain isolated in 1991 in Japan (Osano *et al.*, 1994). More than 18 bla_{IMP} genes coding for different IMP-type β-lactamases have so far been identified in Gram-negative bacteria in many places (Livermore and Woodford, 2000) and are usually present as gene cassettes in class 1 integrons. However, IMP-type MBLs predominate in Asian countries, especially in Japan and Korea. The IMP-1, IMP-2 and IMP-10 have been reported in *P. aeruginosa* isolates

from Japan (Watanabe *et al.*, 1991; Shibata *et al.*, 2003), IMP-1 in *P. aeruginosa* and *A. baumanni* isolates in Korea (Lee *et al.*, 2003), IMP-4 in *Acinetobacter* isolates in Hong Kong (Chu *et al.*, 2001) and in *Citrobacter youngae* isolates in China (Hawkey *et al.*, 2001). IMP-type MBLs isolated outside of Asia include IMP-5 in an *A. baumannii* isolate from Portugal (Da Silva *et al*, 2001), and IMP-7 in a *P. aeruginosa* isolate in Canada (Gibb *et al.*, 2002).

The VIM-type enzymes are another group of MBLs encoded by transferable genes. VIM-1 is the first of the group of enzymes to be detected in a *P. aeruginosa* strain isolated in 1997 in Italy (Lauretti *et al.*, 1999). Although the VIM-type enzymes share less than 30% amino acid identity with the IMP enzymes, they possess the same broad-spectrum substrate profile that includes all β-lactams except aztreonam (Senda *et al.*, 1996a). Most bla_{VIM} genes are also present as gene cassettes in class 1 integrons. More than 11 VIM variants have been described so far. They are commonly found in Europe, for example VIM-1 and VIM-2 in *P. aeruginosa* isolates in Italy (Lauretti *et al.*, 1999; Pallecchi *et al.*, 2001), VIM-4 in *P. aeruginosa* isolates in Greece (Pournaras *et al.*, 2002) and VIM-5 in *P.* aeruginosa solates in Turkey (Bahar *et al.*, 2004). However, there seems to be a worldwide spread of VIM-type enzymes since VIM-7 have been detected in *P. aeruginosa* isolates in the United States (Toleman *et al.*, 2002), VIM-3 in *P. aeruginosa* isolates in Taiwan (Yan *et al.*, 2001) and VIM-2 in *S. marcescens* isolates in Korea (Yum *et al.*, 2002).

SPM-type and GIM-type enzymes are less common MBLs. The SPM-type enzyme, first described in Brazil from a *P. aeruginosa* isolate in 1997, seems to be limited to this country (Toleman *et al.*, 2002). GIM-1 was recovered from *P. aeruginosa* isolates from Germany (Castanheira *et al.*, 2004). Other GIM variants are rarely reported.

The number of oxacillinases (OXA), class D β-lactamases that have carbapenem-hydrolysing properties, have increased considerably in recent years. There are at least seven carbapenem-hydrolyzing OXA enzymes (Walther-Rasmussen and Hoiby, 2006). Of these, OXA-23 in *A. baumannii* isolates has been reported from China (Yu *et al.*, 2004), OXA-25 in *A. baumannii* isolates from Spain (Afzal-Shah *et al.*, 2001) and OXA-50a in *P. aeruginosa* isolates from South Africa (Girlich *et al.*, 2004). These OXA-type carbapenemases are mainly found in these two species. The genes coding for the OXA-type carbapenemases are not integrated into integrons as gene cassettes but are present on the chromosome. Most OXA-type carbapenemases have weak carbapenemase activity only. Carbapenem-resistance in OXA-type carbapenemase-producing strains may be a result of a combination of other resistance mechanisms such as lack of specific porins and overexpression of efflux pump encoding genes (Walther-Rasmussen and Hoiby, 2006).

2.3.4 Efflux pump systems

To decrease the amount of an antimicrobial that accumulates within a cell by efflux pump systems that actively pumps out antibiotic molecules, these systems are responsible for antibiotic resistance. Efflux pump systems actually play an important role in the physiological function since they also pump out numerous dyes, detergents, inhibitors, disinfectants and substances that are harmful to the microorganism. Efflux is a common mechanism of antibiotic resistance since it has been demonstrated in many antibiotic resistant bacteria (Kohler *et al*, 1999).

Antibiotic efflux systems can be classified into five major groups according to the structure of the cytoplasmic membrane transporter proteins: the resistance-nodulation-division (RND) family, the major facilitator (MF) superfamily, the multidrug and toxic compound extrusion (MATE) family, the ATP-binding cassette (ABC) family, and the

small multidrug resistance (SMR) family (Li and Nikaido, 2004; Poole 2004). Transporters of the RND family are antibiotic efflux pumps that are responsible for antibiotic resistance and are the most common among Gram-negative bacteria. These efflux pump proteins are highly homologous and are arranged in three "layers" with drug-proton antiporter of the RND family (MexB, MexD, MexF and MexY) in the cytoplasmic membrane; a membrane fusion protein (MFP) (MexA, MexC, MexE and MexX) in the periplasmic space and a channel-forming protein (also known as outer membrane factor (OMF) (OprM, OprJ and OprN) in the outer membrane, a channel is thus formed connecting the inner and outer membranes. There are four efflux systems that are capable of pumping out multiple drugs including β-lactams. They are MexA-MexB-OprM, MexC-MexD-OprJ, MexE-MexF-OprN and MexX-MexY. These pumps share a similar genetic organization (Figure 1.5) but differ in substrate specificity and regulation (Poole 2000).

The MexA-MexB-OprM (MexAB-OprM) system is constitutively expressed even in wild-type strains and is therefore responsible for intrinsic resistance. Expression of the *mexAB-oprM* operon is repressed by a gene known as *mexR*. Mutation(s) in the *mexR* gene can lead to overexpression of the MexAB-OprM system (Poole *et al.*, 1996). Substrates of this efflux system other than β-lactams (including carbenicillin, piperacillin, ceftazidime, cefepime and aztreonam) include fluoroquinolones, tetracycline and chloramphenicol (Li *et al.*, 1994; Yoneyama, *et al.*, 1997; Saito *et al.*, 1999; Masuda *et al.*, 2000b; Srikumar *et al.*, 2000).

In contrast to the MexAB-OprM system, the MexC-MexD-OprJ (MexCD-OprJ) and MexE-MexF-OprN (MexEF-OprN) systems are not expressed in wild-type strains. The MexCD-OprJ efflux system is regulated by the NfxB repressor (Okazaki and Hirai, 1992). Mutations in the NfxB encoding gene inactivate the NfxB repressor resulting in

overexpression of the MexCD-OprJ system and leading to resistance to fluoroquinolones and β-lactams that include piperacillin, cefepime and meropenem (Poole, 2001). MexEF-OprN is positively regulated by *mexT* since mutations in this gene can lead to overexpression of the *mexEF-oprN* operon, resulting in resistance to fluoroquinolones, trimethoprim, and chloramphenicol (Kohler *et al.*, 1997; Kohler *et al.*, 1999; Maseda *et al.*, 2000).

The MexX-MexY (MexXY) system utilizes OprM since it does not have its own outer membrane protein (Masuda *et al.*, 2000a; Chuanchuen *et al.*, 2001). Its expression can be induced by tetracycline or aminoglycosides (Aires *et al.*, 1999; Mine *et al.*, 1999; Masuda *et al.*, 2000a). The substrates of the MexXY-OprM system include fluoroquinolones, aminoglycosides, and β-lactams that include piperacillin, cefepime, and meropenem (Aires *et al.*, 1999; Mine *et al.*, 1999; Masuda *et al.*, 2000a; Sobel *et al.*, 2003; Wolter *et al.*, 2004). The expression of *mexXY* is repressed by the *mexZ* gene since mutation(s) in *mexZ* lead to overexpression of this system (Vogne *et al.*, 2004).

In contrast to most β-lactams, imipenem is not a substrate of these multidrug efflux systems since it has strong hydrophilic side-chains (Li *et al.*, 1994; Kohler *et al.*, 1999; Pai *et al.*, 2001). Trias and Nikaido (1990) showed by a liposome swelling assay that imipenem penetrates specifically through the outer membrane protein OprD. Increased efflux pump system expression does not affect imipenem susceptibility since influx of the drug through OprD membrane protein is more rapid than extrusion via efflux systems (Trias and Nikaido, 1990).

2.4 Mechanisms of imipenem-resistance in *P. aeruginosa*

The major mechanisms leading to imipenem-resistance in *P. aeruginosa* include production of carbapenem-hydrolyzing enzymes, loss of an outer membrane protein, overexperession of efflux pump systems and hyperproduction of AmpC-β-lactamase (Livermore 1992; Livermore 2000).

. There are five porins in *P. aeruginosa*, OprC, OprD, OprF and OprE (Gotoh *et al.*, 1989). OprD is a 46-kDa outer membrane protein that forms small base-specific pores through which basic amino acids and antibiotics such as carbapenems enter into the cell. However, penicillins, cephalosporins, monobactams and other β-lactams cannot pass through these pores (Huang and Hancock, 1993).

At least two types of imipenem-resistant mutants have been described regarding the outer membrane protein. The major type involves the loss of OprD. Genetic anaylsis of laboratory-derived mutants has shown that the loss of OprD expression is due to deletions in the *oprD* coding region and the upstream promoter region (Yoneyama and Nakae, 1993). Such mutants are resistant only to carbapenems (Lynch *et al.*, 1987).

An alternative mechanism is the decreased production of OprD protein resulting in resistance to both imipenem and other unrelated classes of antibiotics (Cambau *et al.*, 1995; Fukuda *et al.*, 1995). The *nfxC* mutants, also known as norfloxacin mutants, show cross-resistance to fluoroquinolones and imipenem and have reduced expression of OprD (Masuda *et al.*, 1995) as well as overexpression of the MexEF-OprN efflux pump sytem resulting in overproduction of the membrane fusion protein MexE, cytoplasmic membrane protein MexF, and the outer membrane protein OprN (Kohler *et al.*, 1997). Although imipenem cannot be pumped out by the MexEF-OprN pump, overexpression of the *mexEF-oprN* is linked to decreased amounts of the OprD protein (Fukuda *et al.*, 1990; Masuda *et al.*, 1995).

The MexEF-OprN system is positively regulated by MexT which is also a negative regulator for OprD protein expression. Overexpression of *mexEF-oprN* and decreased expression of *oprD* in *P. aeruginosa* are a result of a mutated *mexT* (Kohler *et al.*, 1999; Ochs *et al.*, 1999; Maseda *et al.*, 2004). Sobel and colleagues (2005) showed that disruption of a gene immediately upstream of *mexT* in a clinical isolate of *P. aeruginosa* enhances *mexEF-oprN* expression and decreases OprD production, resulting in the organism becoming resistant to multiple drugs (Figure 1.5).

AmpC β-lactamase is produced constitutively at low levels by most enterobacteria and *P. aeruginosa* (Bush *et al.*, 1995; Livermore 1995), but its production is induced by certain β-lactams such as cefoxitin and imipenem (Giwercman *et al.*, 1990; Wu and Livermore, 1990). The AmpC β-lactamase can also be overproduced due to mutations in the encoding genes resulting in resistance to broad-spectrum cephalosporins and monobactams (Sanders and Sanders Jr, 1987). Mutations in *ampG*, the gene coding for a permease that allows entry of muropeptides into the cytoplasm from the periplasmic space (Korfmann and Sanders, 1989; Lindquist *et al.*, 1993; Hanson and Sanders, 1999), *ampD*, the gene coding for an amidase that cleaves the muropeptides that enter the cytoplasm (Lindberg *et al.*, 1987; Lindquist and Normark, 1987; Jacobs *et al.*, 1995; Hanson and Sanders, 1999) and *ampR*, the gene coding for a protein that regulates *ampC* gene expression are responsible for hyperproduction of AmpC β-lactamase (Lindberg *et al.*, 1987; Kuga *et al.*, 2000). Although imipenem is relatively stable to AmpC hydrolysis, hyperproduction of AmpC β-lactamase is commonly found in imipenem-resistant isolates (Quale *et al.*, 2006). However, other mechanisms responsible for imipenem-resistance are also present in these isolates (Livermore 1995; Yang *et al.*, 1995; Quale *et al.*, 2006).

2.4.1 Prevalence of imipenem-resistant *P. aeruginosa* isolates

Although imipenem is active against many Gram-negative bacteria and has been used successfully to treat infections caused by these bacteria, strains resistant to imipenem have increasingly been reported throughout the world.

In South Africa, the prevalence of imipenem-resistance is alarmingly high at 42% in *P. aeruginosa* and 32% in *A. baumannii* (Brink *et al.*, 2007).

According to a surveillance study of bacterial pathogens in intensive care units in Europe and North America performed during 2000-2002, imipenem-resistance is mainly found among *Acintobacter* spp and *P. aeruginosa* isolates while other Gram-negative bacteria are usually susceptible to imipenem (Jones *et al.*, 2004). The percentage of imipenem-resistant *P. aeruginosa* isolates ranges from 18% in Canada to 28% in Italy while that in *Acintobacter* spp ranges from 2% in Canada to 19% in Italy (Jones *et al.*, 2004).

Imipenem-resistance is also high among *P. aeruginosa* and *Acinetobacter* spp isolates from Asian countries (Wang *et al.*, 2003; Sasaki *et al.*, 2004; Lee *et al.*, 2006). In China, there has been a decrease in the susceptibility of *P. aeruginosa* isolates to imipenem from 88% in 1994 to 77% in 2001(Wang *et al.*, 2003). In Korea, 24% of *P. aeruginosa* isolates and 17% of *Acinetobacter* sp. isolates are resistant to imipenem while the corresponding figure for most of the other Gram-negative bacteria is ≤3% (Lee *et al.*, 2006). In Japan, 22% of *P. aeruginosa* isolates recovered from patients in surgical wards from different regional hospitals are resistant to imipenem (Sasaki *et al.*, 2004).

Imipenem-resistance is also a serious problem in Britain being found in a variety of Gram-negative bacteria other than *P. aeruginosa* and *Acintobacter* spp. During 2001-2002, 3% of *E. coli* isolates, 6% of *Klebsiella* sp isolates, 8% of *M. morganii* isolates, 4% of *Serratia* sp isolates, 7% of *P. aeruginosa* isolates, 30% of *Citrobacter* sp isolates, 35%

of *Enterobacter* sp isolates and all of *S. maltophilia* isolates were resistant to imipenem (Reynolds *et al.*, 2004).

3 Integrons

3.1 Structure and classification

Integrons are elements harboring genes which determine a recombination system that are site-specific. This recombination system enables an integron to recognize and capture genes that are usually found in cassettes known as gene cassettes. An integron contains the gene coding for the enzyme integrase (*int*) that excises and then integrates a gene cassette in the integron, a promoter that governs the expression of the integron, and a gene for the recombination site (*attI*) at which gene cassettes are recognized and inserted (Figure 1.6). The gene cassettes are so-called foreign genetic elements which are integrated and then become part of the integron. Integrons can be present on transposons, plasmids or chromosomes but they themselves are not mobile (Hall and Collis, 1995).

There are two different groups of integrons, the resistance integrons and the super-integrons. The resistance integrons are so-called since the cassettes carry genes that code for resistance to antibiotics and/or disinfectants. They can be present on the chromosome or on plasmids. There are three classes of resistance integrons, namely 1, 2 and 3. Super-integrons are larger than resistance integrons, have only been found on the chromosome, and harbor gene cassettes coding for different functions. There are thus four classes of integrons with the super-integrons belonging to class 4. The integrons are classified according to the homology of the integrase which, in each of the classes, is of 40% - 60% amino acid identity (Carattoli 2001).

3.1.1 Class 1 integrons

The class 1 integron is the most common integron since it is found in many bacterial species. An integron contains two conserved segments, the 5'-conserved segment (5'-CS) and the 3'-conserved segment (3'-CS). The 5'-CS consists of the *intI* gene, the promoter, and the *attI* site while the 3'-CS contains a *qacEΔ1* gene that codes for resistance to quaternary ammonium compounds and a *sul1* gene that codes for resistance to sulphonamides (Stokes and Hall, 1989; Recchia and Hall, 1995).

A gene cassette is made up of a gene and a recombination site, the 59-base element or *attC*, for integration into an integron. The 59-base elements are variable in length and therefore not conserved (Hall *et al.*, 1991; Recchia and Hall, 1995). There is an imperfect inverted repeat with a core region of RYYYAAC near the 3' end of the gene and a core region of GTTRRRY at the other end (Hall *et al.*, 1991; Collis and Hall, 1992). A gene cassette does not contain a promoter and is therefore transcribed from a promoter in the integron.

Only the closed circular form of gene cassette can be integrated into an integron (Collis and Hall, 1992). The integrase interacts with the target recombination sites that are the *attI* site located in the 5'-CS of the integron and the *attC* site located at the 3'-end of each gene cassette (Segal *et al.*, 1999).

Nearly all integron-borne gene cassettes encode resistance to groups of antibiotics that have been in clinical use for a long time such as the sulphonamides. Recently, a number of gene cassettes that encode resistance to the cephalosporins and carbapenems have been reported (Arakawa *et al.*, 1995; Naas *et al.*, 1999).

Class 1 integrons are the most common integrons that have been reported in many clinically important Gram-negative bacteria such as *A. baumannii* (Petersen *et al.*, 2000), *E. coli* (Barlow *et al.*, 2004), *K. pneumoniae* (Lincopan *et al.*, 2005), *P. aeruginosa*

(Toleman *et al.*, 2007), *Samonella* sp (Tamang *et al.*, 2007) and *Shigella* sp (Ahmed *et al.*, 2006).

3.1.2 Other classes of integrons

Class 2 integrons are often found within the Tn7 transposons. The structure of class 2 integrons is similar to that of class 1 integrons. They also consist of an integrase gene (*intI2*) and a recombination site (*attI2*) that is located between the *intI2* gene and the first inserted resistance gene as described for class 1 integrons (Sundstrom and Skold, 1990; Radstrom *et al.*, 1994; Recchia and Hall, 1995; Gravel *et al.*, 1998). The homology of the amino acid sequence of IntI1 and IntI2 are of <50% identity. Although gene cassettes found in class 2 integrons are identical to those in class 1 integrons, the functional IntI2 cannot excise the resistance gene from class 1 integrons and integrate it into a class 2 integron (Hansson *et al.*, 2002). Class 2 integrons have been found in *Acintobacter* sp (Gonzalez *et al.*, 1998), *Shigella* sp (McIver *et al.*, 2002) and *Salmonella* sp (Orman *et al.*, 2002).

The structure of class 3 integrons is comparable to that of both class 1 and class 2 integrons. The amino acid sequences of IntI3 have an identity of 61% to the IntI1. However, class 3 integrons are rarely reported and have only been described in *P. aeruginosa, S. marcescens, Alcaligenes xylosoxidans, P. putida* and *K. pneumoniae* isolates from Japan (Arakawa *et al.*, 1995; Senda *et al.*, 1996b).

The class 4 integron, which is also known as *Vibrio cholerae* super-integron, may harbor as many as 100 gene cassettes, in contrast to resistance integrons in which less than 10 cassettes are present. Many species harbor species-specific super-integrons but

resistance integrons are not species-specific. Super-integrons have been mainly described in *Vibrio* sp and *Shewanella* sp (Clark *et al.*, 2000; Drouin, Heidelberg *et al.*, 2000).

3.2 Integrons in imipenem-resistant *P. aeruginosa*

Class 1 integrons have been described in most of the imipenem-resistant *P. aeruginosa* isolates reported. Genes such as bla_{IMP-1} from Japan (Fukigai *et al.*, 2007), bla_{IMP-7} from Canada (Gibb *et al.*, 2002) and Malaysia (Ho *et al.*, 2002), bla_{IMP-13} from Italy (Toleman *et al.*, 2003), bla_{VIM-2} from France (Poirel *et al.*, 2000), and bla_{VIM-4} from Greece (Pournaras *et al.*, 2002) are all present on a class 1 integron. Most of the class 1 integrons are located on transferable plasmids but some are also found on transposons (Arakawa *et al.*, 1995; Laraki *et al.*, 1999). Class 2 integrons have not been found in *P. aeruginosa* so far while class 3 integrons harbouring bla_{IMP-1} have been reported from Japan (Senda *et al.*, 1996b; Shibata *et al.*, 2003).

4 Objectives

P. aeruginosa is an important cause of nosocomial infections. Due to its intrinsic resistance to many antimicrobial agents, the choice of drugs for treatment of infections caused is very limited. Imipenem has been the most active antibiotic to treat *P. aeruginosa* infections, however strains resistant to it have developed in many places including Hong Kong. This is of serious concern and in order to prevent the spread of imipenem-resistant *P. aeruginosa*, it is important to understand the mechanisms of imipenem-resistance in this organism. The objectives of my project were to:

1. Study the antimicrobial susceptibilities of imipenem-resistant *P. aeruginosa*; and

2. Investigate the mechanisms of imipenem-resistance in these strains including β-lactamase production; outer membrane profiles and mRNA expression of the *oprD* gene; and the effect of the *mexT* gene on the regulation of OprD protein production.

Chapter 2-Materials and methods

1 Materials

All materials used in this study are listed in Table 2.1.

1.1 Bacterial strains

1.1.1 Bacterial strains used in this study

Pseudomonas aeruginosa strains were cultured from clinical specimens of patients in the New Territories East Cluster Hospitals (Prince of Wales Hospital (PWH), Shatin Hospital, Tai Po Hospital, Alice Ho Miu Ling Nethersole Hospital, Bradbury Hospice, Cheshire Home and North District Hospital), during the period of January 2001 and May 2005. These specimens include wound swabs, blood cultures, urine, sputa, body fluid and pus.

All 140 single patient isolates reported by the clinical diagnostic laboratory of PWH to be resistant to imipenem and survived were used for this study. They were stored on nutrient agar slants and kept at room temperature and in glycerol broth at -70°C for further studies.

1.1.2 Reference strains

E. coli ATCC 25922, NCTCC (National Collection of Type Culture) 10418, *S. aureus* ATCC 29213, and *P. aeruginosa* ATCC 27853 were used as control strains in antibiotic susceptibility testing.

β-Lactamases extracted from clinical isolates confirmed to produce SHV-5, P99, TEM-1, TEM-2 and K1 enzymes were used as pI markers in isoelectric focusing experiments.

Clinical isolates confirmed to produce β-lactamases IMP-1, TEM-1, CTX-1, SHV-4, OXA-1, OXA-2 and OXA-10 were used as positive controls for detection of the respective β-lactamase genes. *P. aeruginosa* COL-1 (kindly supplied by Dr. Laurent Poirel, University of Paris-Sud, France) and *P. aeruginosa* 03/9/T104 (kindly supplied by Dr. Kyungwon Lee, Yonsei University College of Medicine, Korea) were used as positive controls for bla_{VIM} and bla_{SIM} gene detection, respectively.

Clinical isolate 4639 confirmed to harbor the *aadA2* gene encoded by class 1 integron gene cassette was used as a positive control for detecting *intI1* and class 1 integron gene cassette. *E. coli* 96K062 and *E. coli* 96K381 (kindly supplied by Dr. JC Lee, Kyungpook National University, Korea) were used as positive controls for *intI2*.

2 Methods

2.1 Subculture of isolates

Strains that were stored on nutrient agar at room temperature or in glycerol broth at -70°C were subcultured on Mueller-Hinton (MH) agar and incubated at 35°C overnight.

2.2 Identification

All strains were initially identified by the API 20E system and their susceptibility to antibiotics was determined by a disk diffusion method (CLSI, 2005) in the clinical microbiology laboratory of PWH. After subculture, their identity was reconfirmed by the oxidase test (Koneman *et al.*, 1997).

2.3 Antibiotic susceptibility testing

The susceptibilities of the strains to 19 antibiotics (Table 2.2) were tested by determining the minimum inhibitory concentration (MIC) using an agar dilution method (CLSI, 2005).

2.3.1 Preparation of antibiotic plates

Antibiotic powder was weighed (calculated according to the following formula) and dissolved in an appropriate solvent (Table 2.2) to make up a stock solution of 50,000 mg/l or 5,000 mg/l and kept in a -70°C freezer.

$$Weight\ (mg) = \frac{Volume\ (ml) \times Concentration\ (mg/L)}{Potency\ (units/mg)}$$

The antibiotic stock solution was diluted in a series of 1:10 dilution with distilled water. MH agar was prepared according to the manufacturer's instruction. An appropriate volume of an antibiotic was added to MH agar in order to prepare plates with the desired final concentration. Agar plates containing serial two-fold dilutions of antibiotics in the concentration range as listed in Table 2.2 were prepared. All antibiotic plates were kept in a 4°C cold room and used within two days after preparation.

2.3.2 Inoculation of antibiotic plates

Several overnight single colonies on MH agar were emulsified in 1.5 ml of 0.85% sterile saline to a turbidity equivalent to that of a 0.5 McFarland standard (approximately 1.5×10^8 cfu/ml). The bacterial suspensions were further diluted 10-fold with 0.85% sterile saline and were then inoculated onto antibiotic-containing agar by the MIC2000 inoculator which deposited approximately 1 μl onto the agar surface. The final inoculum

on the agar would then be approximately 10^4 cfu per spot. The plates were incubated at 35°C for 16 to 20 hours.

2.3.3 Determination of minimum inhibitory concentration (MIC)

The MIC of an antibiotic for a strain is the lowest concentration of antibiotic that inhibits its growth. A strain was regarded as resistant to an antibiotic if its MIC was greater than the breakpoint concentration for susceptible strains according to the CLSI (CLSI, 2005). The MIC_{50} and MIC_{90} are the concentrations of an antibiotic that inhibit growth of 50% and 90% of the tested strains, respectively.

2.4 Phenotypic detection of metallo-β-lactamase (MBL) production

P. aeruginosa strains that had an imipenem MICs ≥8 mg/L were screened for metallo-β-lactamase (MBL) production by the imipenem-EDTA disk method (Yong *et al.*, 2002). A *P. aeruginosa* strain confirmed to produce IMP (a MBL) and one that does not produce MBLs, ATCC 27853, were used as controls.

2.4.1 Preparation of inoculum

Several overnight colonies on MH agar plate were emulsified in 1 ml of sterile saline to a turbidity equivalent to that of a 0.5 MacFarland standard.

2.4.2 Imipenem-EDTA disk test

The bacterial suspension was spread evenly onto the surface of an MH agar using a sterile cotton swab. Two 10-μg imipenem disks were placed on the surface of the bacterial lawn about 40 mm apart from each other. Four microliters of a 0.5 M EDTA

solution (prepared by dissolving 186.1 g of disodium EDTA·$2H_2O$ in 1,000 ml of distilled water and adjusting it to pH 8.0 by NaOH (Sambrook *et al.*, 2001), equivalent to 750 µg) were added to one of the disks. The plates were incubated at 35°C for 16 to 20 hours.

2.4.3 Determination of MBL strains

After incubation, the diameters of the zone of inhibition were measured. MBLs require zinc for their catalytic activity and are inhibited by EDTA (Bush, Jacoby and Medeiros, 1995; Buynak *et al.*, 2004). A difference in zone diameter of ≥8 mm indicated production of MBL by the tested strain (Yong *et al.*, 2002).

2.5 Extraction of crude β-lactamase

Crude β-lactamase was extracted from imipenem-resistant strains by ultrasonication.

P. aeruginosa strains were grown on MH agar at 35°C overnight. A loopful of this bacterial culture was emulsified in 600 µl of 0.1 M sodium phosphate buffer (pH 7.0), centrifuged at 5,000 x g for 5 minutes, the supernatant removed, the cell pellet resuspended in 400 µl of distilled water and centrifuged again at 5,000 x g for 5 minutes. The cell pellet was finally resuspended in 30 µl of distilled water and then sonicated in a sonicator (MSE Soniprep 150) at an amplitude of 10 microns for 15 seconds for 4-6 times with the sample tube chilled in ice.

2.5.1 Detection of β-lactamase production

One microliter of 500 mg/L of nitrocefin was added to the crude extract (prepared as described in 2.5 above) on a porcelain plate and mixed with a wire loop. Presence of β-lactamase was indicated by nitrocefin changing from yellow to red within 1-2 minutes. All crude extracts giving a positive nitrocefin reaction were stored at -70°C for future use.

2.6 Isoelectric focusing (IEF)

The isoelectric points (pIs) of imipenem-resistant isolates were estimated by IEF on a precast polyacrylamide gel of pH 3 to 10.

2.6.1 Set up of electrophoresis equipment

The precast polyacrylamide gel was removed from its package and placed on the Multiphor II electrophoresis unit. The cathode and anode electrode strips were prepared by soaking in 1 mol/L of sodium potassium and 1 mol/L of phosphoric acid, respectively. They were then applied to the edges of the gel. Pieces of sample application paper were placed at 40 mm from the cathode (instead of 10 mm as suggested by the manufacturer) to give better separation of protein bands and at a distance of about 5 mm from each other.

2.6.2 Sample application and instrument preparation

Twenty microliter of a crude enzyme extract was applied to each piece of sample application paper. The electrode holder was placed on the Multiphor II unit and the electrodes were aligned in the center of the electrode strips. The two electrodes were connected to the base unit and the safety lid was placed in position.

2.6.3 Running conditions

The Multiphor II electrophoresis unit was connected to the MultiTemp II thermostatic circulator to give a running temperature of 10°C during electophoresis. The gel was run at 1,500 V, 50 mA and 30 W for about 1.5 hours.

2.6.4 Detection of β-lactamase

After electrophoresis, the gel was overlaid with a piece of filter paper that was soaked with 4 ml of 500 mg/L of nitrocefin. Presence of β-lactamase was indicated by appearance of red bands within 1-2 minutes.

2.6.5 Determination of isoelectric point (pI)

Crude extracts prepared from strains harbouring β-lactamase genes bla_{SHV-5} (pI 8.2), bla_{P99} (pI 7.8), bla_{K1} (pI 6.5), bla_{TEM-2} (pI 5.6) and bla_{TEM-1} (pI 5.4), were used as pI markers. These markers were included on every gel with tested samples. The distance of the β-lactamase bands from the cathodic edge of the gel was measured. A graph of distance (Y-axis) versus pI of pI markers (X-axis) was plotted. The pI of the β-lactamase of the tested sample could be read off from the graph.

2.7 Bioassay of imipenem hydrolysis

This was used to test the ability of β-lactamases of our imipenem-resistant isolates to hydrolyse imipenem.

2.7.1 Preparation of inoculum and plate

An imipenem susceptible standard strain (*E. coli* ATCC 35218) was used to inoculate an MH agar plate. Several overnight single colonies on MH agar were

emulsified in 1.5 ml of 0.85% sterile saline to a turbidity equivalent to that of a 0.5 McFarland standard. The bacterial suspension was spread evenly onto the surface of an MH agar by a sterile cotton swab. Five holes, 20 mm apart from each other, were made on each agar plate with a sterile hole puncher.

2.7.2 Preparation and incubation of sample mixtures

Crude β-lactamase extract (20 µl) was mixed with 20 µl of 1,000 mg/L (0.5 mg/L) of imipenem in a 0.5 ml eppendorf tube. Crude extracts prepared from strains producing IMP-1 and OXA-2 were used as positive and negative controls, respectively. The mixture was then incubated at 37°C for 2 hrs. Mixtures containing 20 µl of distilled water instead of β-lactamase extract and imipenem and 20 µl of distilled water and the same amount of imipenem but was denatured by boiling at 100°C for 30 minutes were also incubated under the same condition.

2.7.3 Application of sample mixtures

After incubation, the mixtures were vortexed and centrifuged briefly. Twenty microliters of each of the mixtures prepared as described in 2.7.2 above were added to each hole in the agar. The plate was then incubated (with the lid facing upwards) at 35°C overnight.

2.7.4 Determination of imipenem hydrolysis

The diameter of the following zones of inhibition was measured after incubation:

1. That around the hole containing the crude extract of the tested strain and 0.5 mg/L of imipenem;

2. That around the hole containing the crude extract of a imipenem hydrolyzing strain, IMP-1, and 0.5 mg/L of imipenem (positive control);
3. That around the hole containing the crude extract of a imipenem non-hydrolyzing and 0.5 mg/L of imipenem (negative control);
4. That around the hole containing distilled water and 0.5 mg/L of imipenem; and
5. That around the hole containing distilled water and 0.5 mg/L of denatured imipenem.

Imipenem in the mixture was regarded as active if the diameter of the zone of inhibition around the hole was ≥15 mm, the zone diameter interpretive standard for susceptible strains (CLSI, 2005).

2.8 Detection of β-lactamase genes

2.8.1 Polymerase chain reaction (PCR)

Presence of β-lactamase genes bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{TEM}, bla_{CTX}, bla_{SHV}, and bla_{OXA} in imipenem-resistant isolates were screened by PCR.

2.8.2 Preparation of DNA template

DNA template was prepared by emulsifying one to two overnight colonies on a MH agar plate in 200 µl of double distilled water and heating at 100°C in a boiling water bath for 10 minutes. The DNA template was stored at -20°C until further use.

2.8.3 Preparation of PCR master mix

The PCR master mix in a total volume of 25 µl was prepared as listed in Table 2.3. The DNA template of the reference strains that were positive for the genes amplified were used as positive controls. Nuclease-free water as template instead of sample DNA was used as a negative control. Positive and negative controls were included in each set of PCR.

The primers used in detecting β-lactamase genes are listed in Table 2.4.

2.8.4 PCR running conditions

Amplification was performed in a thermal cycler (Gene Amp PCR system 9600) using the following conditions: 7 minutes of denaturation at 95°C followed by 35 cycles of 1 minute at 95°C, 1 minute of annealing at 52°C and 1 minute of extension with a final extension of 7 minutes at 72°C.

2.8.5 Agarose gel electrophoresis

PCR products were separated by gel electrophoresis in a GNA 100 electrophoresis apparatus (Amersham Pharmacia Biotech). A 1% agarose gel was prepared by adding 500 mg of agarose powder to 100 ml of 0.5 X TBE buffer (0.045 M Tris-borate, 0.001M EDTA) and then heating in a microwave oven until the gel was completely melted. The gel was poured into a gel tray with a comb placed 15 mm from one end of the gel tray. The setup was put in place until the gel was completely set. The gel was then placed into the gel tank containing 0.5 X TBE buffer. PCR product (5 µl) was mixed with 1 µl of 6x loading buffer (0.25% bromophenol blue and 15% ficoll type 400) and then loaded into the well of the gel. Φ174/*Hae*III digest was used as molecular weight marker. The gel was electrophoresed for 1 hr at 110 V.

After electrophoresis, the gel was stained with 0.5 mg/L of ethidium bromide for about 30 minutes, rinsed with distilled water and visualized and photographed under UV transillumination using a gel documentation system (Thermal Imaging system CTI-500).

2.8.6 DNA sequencing

PCR products were purified before sending off to Macrogen for DNA sequence determination.

PCR products were purified using the MicroSpin S-300 HR columns. To prepare the column for use, the resin in it was resuspended by vortexing, the bottom closure was snapped off and the column was then spun for 1 minute at 735 x g. PCR product (about 25 µl) was applied to the column, centrifuged at 735 x g for 2 minutes and the purified product was collected in a 1.5 ml eppendorf tube. DNA concentration in the purified product was checked by gel electrophoresis as described in section 2.8.5.

The purified PCR products were sent to Macrogen Inc. for DNA sequence determination by the single extension method (Sambrook *et al.*, 1989). Results from Marcogen were then analyzed by the Chromas 1.42 software.

The Basic Local Alignment Search Tool, BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), was used to find regions of similarity between sequences. The program compares nucleotide sequences to sequence databases and calculates the statistical significance of matches.

2.9 Detection and characterization of integrons

2.9.1 PCR

Presence of *intI1* and *intI2* in the imipenem-resistant isolates was screened by Components of the PCR mix are shown in Table 2.5 and primers IntI1 and IntI2 used are shown in Table 2.6. DNA extracted from strains harboring *intI1* and *intI2* was used as positive controls and nuclease-free water in place of DNA template was used as the negative control.

PCR running conditions consisted of 7 minutes of denaturation at 95°C followed by 35 cycles of 1 minute at 95°C, 1 minute of annealing at 56°C for *intI1* and 60°C for *intI2*, 1 minute of extension with a final extension of 7 minutes at 72°C. PCR products were separated by agarose gel electrophoresis as described in section 2.8.5.

2.9.2 DNA sequencing

PCR products were purified and their DNA sequences determined as described in section 2.8.6.

2.10 Detection and characterization of gene cassettes

2.10.1 PCR

Presence of gene cassettes and the 3'-conserved segments in the imipenem-resistant isolates that harbor class 1 integron was screened by PCR using two sets of primers as shown in Table 2.7. Components of the PCR mix are shown in Table 2.8 and Table 2.9. DNA extracted from strains harboring class 1 integron gene cassette was used as positive controls and nuclease-free water in place of DNA template was used as the negative control.

PCR running conditions for amplifying gene cassettes consisted of 5 minutes of denaturation at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds of annealing at 57°C, 3 minutes of extension with a final extension of 7 minutes at 72°C.

PCR running conditions for amplifying 3'-conserved segments consisted of 5 minutes of denaturation at 94°C followed by 35 cycles of 1 minute at 94°C, 1 minute of annealing at 55°C, 1 minute of extension with a final extension of 7 minutes at 72°C.

PCR products were separated by agarose gel electrophoresis as described in section 2.8.5.

2.10.2 DNA sequencing

PCR products were purified and their DNA sequences determined as described in section 2.8.6.

2.11 Investigation of membrane permeability

2.11.1 Extraction of outer membrane proteins (OMP)

Several overnight colonies on a MH agar was selected and emulsified in 600 µl of 10 mM Tris-HCl (pH 8.0) and centrifuged for 10 minutes at 5,000 x g. The cells were washed with 10 mM Tris-HCl (pH 8.0) and resuspended in 400 µl of the same buffer. Crude OMP extracts were prepared by sonication using Soniprep 150 at an amplitude of 10 microns for 15 seconds for 4-6 times with the samples tubes placed in ice.

The extracts were then centrifuged at 3,800 x g for 10 minutes. The supernatant was aspirated and the total volume made up to 1 ml with 10 mM Tris-HCl (pH 8.0). Triton-100 (100%, 10 µl) was added, the mixture incubated for 30 minutes at 37°C and then centrifuged at 38,000 x g for 30 minutes at 4°C in a Centrikon T-2080 centrifuge.

The pellet containing OMPs was resuspended in 400 µl of 10 mM Tris-HCl (pH 8.0) and stored at -20°C until use.

2.11.2 Quantification of OMP

The crude OMP extracts were quantified by the microplate procedure of Bio-Rad BCA™ Protein Assay.

2.11.3 Preparation of the albumin standards and working reagents

Different concentrations of albumin standards (0 mg/L, 25 mg/L, 125 mg/L, 250 mg/L, 500 mg/L, 750 mg/L, 1,000 mg/L, 1,500 mg/L and 2,000 mg/L) were prepared by diluting the stock albumin standard (2 mg/L in 0.9% saline and 0.05% sodium azide) with appropriate volumes of distilled water.

A duplicate sample was prepared for each standard and unknown. The albumin standard (20 µl) and the tested samples (20 µl) were added to each well of a microplate that contained 180 µl of working reagents. Working reagents were prepared by mixing 50 parts of BCA™ reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and 1 part of BCA™ reagent B (containing 4% cupric sulfate). The mixture in the wells of the plate was mixed thoroughly by placing the plate on a plate shaker (Thermo Electron Corporation) to be shaken gently for 30 seconds. The plate was then covered with a plastic lid and incubated at 37°C for 30 minutes.

After incubation, the absorbance of each well was measured at 560 nm using a plate reader (Multiskan ascent) after the plate was shaken for 30 seconds to mix the mixtures in wells.

2.11.4 Determination of protein concentration

A curve of the concentration of albumin standards as the Y-axis and the absorbance as the X-axis was drawn. The concentration of the unknown samples was read from the curve.

2.12 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The extracted OMPs were separated and their size determined by SDS-PAGE.

2.12.1 Sample preparation

The extracted OMPs (17.5 µl) were mixed with 1 X (1.25 µl) of XT-reducing agent and 1 X of XT-sample buffer (6.25 µl) to make up to a total volume of 25 µl. The mixtures were denatured at 100°C for 10 minutes in a thermal controller (Gene Amp PCR system 9600).

2.12.2 Gel preparation and sample application

A criterion^{TM} XT precast gel was placed in the criterion ^{TM} cell medium gel tank. The 20X XT MOPS running buffer was first diluted 1:20 with distilled water and was used to fill up the tank. Samples (25 µl) were then applied to each well of the gel. The SDS-PAGE molecular weight standard (Prestained SDS-PAGE standards broad range) was used as a control. The gel was electrophoresed using a PowerPac HC^{TM} for 1.5 hrs at 150 V and 0.3 A.

2.12.3 Staining and destaining of the gel

After electrophoresis, the gel was removed from the gel tank and then rinsed with 200 ml of distilled water before staining with 50 ml of coomassie blue G-250 with shaking for 2 hrs. After staining, the coomassie blue was rinsed off with 200 ml of distilled water and then soaked in 50 ml of destaining solution (10% acetic acid, 30% methanol and 60% distilled water) for 2 hrs. After destaining, an image of the gel was photographed using Chemidoc XRS and analyzed using Quantity One software.

2.13 Expression of the *oprD* gene

The expression level of the outer membrane encoding gene *oprD* was quantified by real time RT-PCR.

2.13.1 Extraction of RNA

Total RNA from the strains was extracted using the RNeasy Mini kit with slight modifications.

An overnight single colony on a MH agar plate was inoculated in 2 ml of Luria-Bertani (LB) broth and incubated at 37°C for 4 hrs with shaking after which 1 ml was pipetted into a 1.5 ml eppendorf tube. Bacterial cells were harvested by centrifuging at 5,000 x g for 5 minutes at 4°C using an Eppendorf centrifuge. The supernatant was decanted followed by aspiration. The cell pellet was loosened by flicking the bottom of the tube and was resuspended thoroughly in 100 µl of lysozyme-containing TE buffer (1 mg/L of lysozyme in 10 mM Tris-HCl, 1 mM EDTA; pH 8.0) by vortexing. This was incubated at room temperature for 15 minutes.

β-mercaptoethanol (β-ME) (10 µl/ml of Buffer RLT) (350 µl) was added to the sample and thoroughly mixed by vortexing. Ethanol (95%) (250 µl) was added to the lysate and thoroughly mixed by pipetting up and down. About 700 µl of the sample was applied to the RNeasy mini column which was placed in a 2-ml collection tube. The column was centrifuged for 15 seconds at 8,000 x g.

After centrifugation, the flow-through was discarded and 700 µl of Buffer RW1 was added to the RNeasy column and centrifuged for 15 seconds at 8,000 x g to wash the column. The flow-through was discarded and 500 µl of Buffer RPE was added to the RNeasy column. It was centrifuged for 2 minutes at 8,000 x g to dry the RNeasy silica-gel membrane.

The RNeasy column was transferred to a new 1.5-ml collection tube and 40 µl of RNase-free water was directly pipetted onto the RNeasy silica-gel membrane. RNA was eluted from the tube by centrifuging for 1 minute at 8,000 x g.

2.13.1.1 Inhibition of RNase degradation

To prevent degradation of the extracted RNA by RNase, 1 µl (1 U) of Recombinant RNasin® Ribonuclease inhibitor was added to 40 µl of RNA sample.

2.13.1.2 Removal of DNA

To remove any DNA that might be present in the RNA sample, 1 U of DNase I was added to 2 µl of an RNA sample and RNase-free water was added to make up to a total volume of 10 µl. The mixture was incubated at room temperature for 15 minutes. The DNase activity was inactivated by adding 1 µl of 25 mM EDTA (RNase-free) and incubating for 15 minutes at 65°C. Presence of DNA in the RNA sample was checked by

PCR using primers specific for the housekeeping gene *rpoD* (as described in section 2.8.5).

2.13.1.3 Quantification of RNA samples

The amount of RNA was measured by using NanoDrop ND-1000 according to the manufacturer's instructions. Two microliters of RNase-free water were applied onto the measurement pedestal and was measured. This measurement was taken as a blank. When the measurement was done, the blank sample was wiped off and RNA samples (2 µl each) were similarly measured. Results were analyzed by the ND-1000 3.3.0 software. The absorbance was measured at both 260 nm and 280 nm and the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA. A ratio of about 2.0 was accepted as pure for RNA.

2.13.2 Real-time RT-PCR

Real-time RT-PCR was performed using the ABI PRISM 7700 Sequence Detection System. The method of relative quantitation of gene expression was used to quantify differences in the expression level of a target gene between different samples with reference to the endogenous control.

In this study, the target gene was *oprD* and the endogenous control was *rpoD*, the housekeeping gene that is consistently expressed in *P. aeruginosa* (Savli *et al.*, 2003). The reporter dye of the probes was FAM™ and no quencher dye was used. The primers and probes used are shown in Table 2.10.

2.13.2.1 Preparation of real-time RT-PCR mixtures

TaqMan EZ RT-PCR Core Reagents kit was used to prepare the real-time PCR mixture in a total volume of 25 µl (Table 2.11).

2.13.2.2 Real-time RT-PCR running conditions

The thermal cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Each RNA sample was done in duplicate.

2.13.2.3 Construction of relative standard curves

RNA (1 µg) from *P. aeruginosa* ATCC 27853, which was used as the calibrator sample, was diluted 10-, 100-, 1,000- 10,000-fold with RNase-free water. RNA of known concentrations was run along with the tested samples for each reaction. The diluted RNA samples were used to construct a relative standard curve for *oprD* and *rpoD* of the logarithm of RNA concentrations versus C_T, where C_T is the number of cycles of a reaction when the fluorescence generated is at the threshold. The amount of RNA of the *rpoD* and *oprD* genes in the tested samples could then be extrapolated from the respective standard curves.

2.13.3 Analysis of real-time RT-PCR results

Results were analyzed using Sequence Detection System software version 1.7. The baseline range and threshold level were adjusted to achieve the highest correlation coefficient of the standard curve. The C_T values from the duplicated samples were averaged and the copy number (the amount of RNA) was extrapolated from the standard

curve. The test samples and *P. aeruginosa* ATCC 27853 were normalized by *rpoD* to calculate a normalized *oprD* value using the following formulae:

Normalized *oprD* (test sample) = *oprD*/*rpoD*

Normalized *opoD* (*P. aeruginosa* ATCC 27853) = *oprD*/*rpoD*

The normalized *oprD* value of the test sample was then divided by that of *P. aeruginosa* ATCC 27853 to give the fold-difference in target quantity between the test sample and the calibrator sample. The normalized *oprD* of *P. aeruginosa* ATCC 27853 was assigned a value of 1. The expression of *oprD* of samples relative to *P. aeruginosa* ATC 27853 less than 1 was regarded as reduced expression.

2.14 Characterization of outer membrane protein regulator *mexT*

2.14.1 PCR

The outer membrane protein regulator *mexT* was amplified by PCR that was performed in a total volume of 25 µl (Table 2.12). DNA extract of *P. aeruginosa* ATCC 27853 was used as the positive control and nuclease-free water instead of DNA template was used as the negative control.

The primers used for PCR is listed in Table 2.13.

PCR running conditions consisted of 7 minutes of denaturation at 95°C followed by 35 cycles of 1 minute at 95°C, 1 minute of annealing at 50°C and 1 minute of extension with a final extension of 7 minutes at 72°C. PCR products were separated by agarose gel electrophoresis as described in section 2.8.5.

2.14.2 DNA sequencing

PCR products were purified and the DNA sequence determined as described in section 2.8.6.

Chapter 3-Results

1 Prevalence of imipenem-resistant *P. aeruginosa* isolated from patients in hospitals of the New Territories East Cluster (NTEC) from 2001 to 2005

A total of 140 non-duplicate isolates were cultured from clinical specimens of patients in the NTEC hospitals during the period of January 2001 - May 2005, with 13 isolated in 2001, 35 in 2002, 39 in 2003, 28 in 2004 and 25 in 2005. The majority of the clinical specimens were from the respiratory tract (62.1%) including sputa (32.1%) and bronchial and tracheal aspirates (30%) (Table 3.1). The other specimens were urines (19.2%), wound swabs (12.1%) and body fluids, pus and miscellaneous specimens that were not specifically indicated (6.4%).

1.1 Age and sex distribution of patients

The age and sex distribution of patients are shown in Table 3.2. More than 60% were aged 61 or older. The average age was 64 and the median was 68. Only 1.4% of patients were aged 0 - 9 years and 31.4% were aged 10 - 60 years. The male to female patient ratio was 1:0.43.

1.2 Antimicrobial susceptibilities

1.2.1 Susceptibility to carbapenems

The imipenem MICs ranged from 8 mg/L to 128 mg/L with the MIC_{50} and MIC_{90} being 16 mg/L and 32 mg/L, respectively. Two isolates had an imipenem MIC of 64 mg/L and one 128 mg/L, both of which were isolated during 2002.

Only 78.2% of these imipenem-resistant strains were resistant to 4 mg/L of meropenem, with an MIC range of 8 mg/L - >128 mg/L, MIC_{50} of 16 mg/L and MIC_{90} of >128 mg/L. The percentage of meropenem resistance ranged from 74.3% to 76.9% during 2001 - 2004 but was 88% in 2005. However, this was not significantly different.

1.2.2 Susceptibility to other β-lactams

The percentage of resistance to cefotaxime, ceftriaxone, ceftazidime and aztreonam was 96.7%, 97.4%, 45.7% and 63%, respectively (Table 3.3). One hundred thirty-nine out of the 140 strains tested (~100%) were resistant to cefoxitin. The proportion of strains resistant to piperacillin was 15.4% - 28.6% during 2001 - 2004 but it increased to 40% in 2005 while the corresponding figures for piperacillin in the presence of tazobactam were 15.4% - 25% and 36%. Resistance to ticarcillin during the period 2001 - 2005 was 55.6% and that to ticarcillin in the presence of clavulanic acid was 47.3%. Resistance to cefepime was 69.2% - 60.7% during 2001 - 2004 but was 80% in 2005. The percentage resistance to ceftazidime was 61.5% in 2001 but decreased to 31.4% in 2002. It then increased to 48.7% - 44% during 2003 - 2005.

1.2.3 Susceptibility to aminoglycosides and fluoroquinolones

Only 6.7% of the strains tested were resistant to tobramycin while 11.8% - 22.7% were resistant to the other three aminoglycosides tested (amikacin, gentamicin and netilmicin). There was a decreasing trend of resistance to all four aminoglycosides tested during the period 2001-2005 with resistance to tobramycin dropping to 0% and that to the other three aminoglycosides to 8% - 12% in 2005 (all p values >0.05).

Resistance to the fluoroquinolones tested (levofloxacin, norfloxacin and ofloxacin are ranged from 13.3% to 28%. The percentage of levofloxacin resistance increased from 7.7% in 2001 to 20% 2005. The percentage of norfloxacin resistance was highest (21.4%) in 2004 and percentage of resistance to ofloxacin was lowest (14.3%) at 2002 during the period 2001 - 2005.

1.2.4 Resistance patterns

The resistance patterns of the 140 isolates tested are listed in Table 3.4. There were a total of 71 different resistance patterns, ranging from resistance to two to 19 antibiotics. Most of the isolates (14.3%) were resistant to four antibiotics, followed by those resistant to 10 (12.9%) and 12 (12.1%) antibiotics. More than 50% (53.1%) were resistant to ten or more antibiotics. Only one isolate was resistant to 19 antibiotics. The most common resistance pattern was resistance to imipenem (IMP), cefotaxime (CTX), cefoxitin (CX) and ceftriaxone (CRO) (14.3%) followed by resistance to imipenem, meropenem (MEM), cefotaxime and ceftriaxone (7.1%).

2 Phenotypic detection of metallo-β-lactamase (MBL) producing strains

This was performed by the imipenem-EDTA disk method as described in section 2.4.

The zone of inhibition around the imipenem disk for the strain harboring the $bla_{\text{IMP-1}}$ gene that was used as a MBL positive control was 6 mm which was 12 mm smaller than that around the disk with 10 μg of imipenem and 750 μg of EDTA, while the corresponding figures for the MBL-negative strain ATCC 27853 *P. aeruginosa* was 21

mm and 1 mm (Figure 3.1). All isolates tested gave an increase in the diameter of the inhibition zone around the disk containing imipenem and EDTA as compared to that around the imipenem disk of 2 mm - 12 mm (Table 3.5). Using the criterion of ≥8 mm difference to indicate production of MBL by a strain (Yong *et al.*, 2002), 20 strains were MBL positive. Two of these had an imipenem MIC of 64 mg/L and one, 128 mg/L. For the MBL-negative strains, 102 (85%) had imipenem MICs of 8 mg/L - 16 mg/L.

2.1 Characterization of β-lactamases

2.1.1 Production of β-lactamases

One hundred twenty-five (89.3%) of the 140 isolates tested were screened positive for β-lactamases.

2.1.2 Determination of isoelectric points of β-lactamases

???i???soelectric focusing was preformed on crude β-lactamase extracts from the 125 β-lactamase producing strains. Ninety-one of these isolates each produced one to four β-lactamases of pIs ranging from 7.6 to 8.2 and of 21 different combinations (Table 3.6). The majority of the isolates (75.8%) produced only one β-lactamase and 6.6% - 7.7% produced two to four β-lactamases. Of the isolates that produced two β-lactamases, one each produced β-lactamases of four different combinations and two of another combination while of those that produced three β-lactamases, one and two each produced β-lactamases of three different combinations. The seven isolates that each produced four β-lactamases were of three different combinations. A β-lactamase of pI 7.8 was the most

common β-lactamase (24.3%). β-Lactamases of pIs 7.9, 8.0 and 8.2 were each produced by 23.1%, 28.6% and 24.2% of isolates, respectively.

2.2 Imipenem hydrolysis by β-lactamases

This was performed by a conventional bioassay (Figure 3.2). The activity of imipenem was first tested by comparing the diameter of the zone of inhibition around the well containing imipenem on a lawn of *E. coli* ATCC 35218 with that around the well containing the same amount of imipenem that had been heat-denatured. There was no zone of inhibition around the well containing the denatured imipenem nor around that containing a mixture of the same amount of imipenem and a crude β-lactamase extract from an IMP-1-producing strain (positive control) while the zone of inhibition around the well containing imipenem was of a diameter of 30 mm in average for all tested strains. The diameter of the zone of inhibition around the well containing a mixture of a crude β-lactamase extract from a TEM-1-producing strain and 0.5 mg/L of imipenem was 28 mm and that around the well containing a mixture of a crude extract of β-lactamase from each of the imipenem resistant strains and 0.5 mg/L of imipenem was 28 mm - 30 mm.

2.3 Detection of β-lactamase genes

PCR with specific primers was used to detect presence of β-lactamase genes bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{TEM}, bla_{CTX}, bla_{SHV}, and bla_{OXA} in all the imipenem-resistant isolates. Only two strains produced PCR products of the expected size, a 760-bp product from one strain (isolate no. 2244) using primer pairs OXA-10F and OXA-10R and a 700-bp product from another (isolate no. 4638) using primer pairs OXA-2F and OXA-2R (Figure 3.3).

2.3.1 DNA sequence determination

Determination of the DNA sequence of the PCR product from isolate no. 2244 showed that it was identical to that of the OXA-10 β-lactamase gene (gene bank accession no. AM 392427) (Appendix 1).

Determination of the DNA sequence of the PCR product from isolate no. 4639 showed that it was identical to that of the β-lactamase OXA-3 gene (gene bank accession no. L07945) (Appendix 2).

3 Detection and characterization of integrons

Presence of integrons in all imipenem-resistant isolates was screened by detecting genes *intI1* and *intI2* by PCR. Ninety-four (67.1%) strains harbored the *intI1* gene while none harbored the *intI2* gene.

DNA sequence determination of these 94 PCR products showed that they were identical to that of *the intI* of class 1 integron (gene bank accession no. EF577407) (Appendix 3).

3.1 Antibiotic susceptibility and resistance patterns of isolates harboring integrons

The antibiotic susceptibilities and resistance patterns of *P. aeruginosa* isolates with a class 1 integron were compared to those of isolates without a class 1 integron (Tables 3.7 and 3.8). There was no significant difference between the proportion of integron-positive and integron-negative isolates that were resistant to all the antibiotics tested except amikacin ($p<0.05$).

All integron-positive isolates were resistant to two to 18 antibiotics in 53 different patterns (Table 3.8). Most of the isolates (17%) were resistant to four antibiotics while 11.7% and 10.6% were resistant to five and 10 antibiotics, respectively. The most common resistance pattern was IMP CTX CX CXO (17%) and followed by IMP MEM CTX CX CRO (8.5%).

4 Detection of gene cassettes

One or two amplicons were produced after amplification of DNA from 74 out of the 94 integron-positive isolates using primers specific for gene cassettes of the class 1 integron. A 200-bp amplicon was produced from 34 isolates (45.9%), a 1,000-bp from 32 isolates (47.3%) and a 800-bp and a 900-bp from four isolates (5.4%) each (Figure 3.4).

No resistance gene was present in all the 200-bp amplicons. The *aadA1* (Gene bank accession no AF313472) (Appendix 4), *aadA2* (Gene bank accession no DQ091178) (Appendix 5), *aadA6* (Gene bank accession no DQ091179) (Appendix 6), bla_{OXA-3} and bla_{OXA-10} genes were identified in 40 out of 74 isolates. The distribution of resistance genes in gene cassettes is shown in Table 3.9. The *aadA1* was present in three amplicons, *aadA2* was present in 28 amplicons, *aadA6* was present in seven amplicions The bla_{OXA-3} and bla_{OXA-10} gene each were present in a 1,000-bp amplicon. The *aadA2* was the most common resistance gene identified in the isolates (70%). There were six different structures of integron among the integron-positive isolates (Figure 3.5).

5 Outer membrane permeability

5.1 Outer membrane protein profiles

The sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the outer membrane proteins was performed on 28 strains that were selected according to their imipenem susceptibility. These were four with imipenem MICs of 64 mg/L and 128 mg/L, 16 with imipenem MICs of 16 mg/L and 32 mg/L and eight with an imipenem MIC of 8 mg/L. A protein of ~46 kDa, a size that was similar to that of the OprD outer membrane protein, was present in the *P. aeruginosa* ATCC 27853 standard strain and in 19 (68%) of the 28 strains tested (Figure 3.6).

5.2 mRNA expression of the *oprD* gene

All 28 isolates were subjected to real-time RT-PCR to determine the mRNA expression of the *oprD* gene and results are shown in Table 3.10. Nine isolates were found to have *oprD* expression reduced as compared to that in *P. aeruginosa* ATCC 27853. Eight of them exhibited resistance to meropenem. The 46 kDa protein was present in two of these isolates (isolate no. 2389 and isolate no. 1287) with reduced *oprD* expression but was absent in two isolates (isolate no. 2132 and isolate no. 3767) that had 1.1-fold and 4.9-fold *oprD* expression.

The mode and median MICs of imipenem were 32 mg/L for isolates with reduced *oprD* expression while they were 16 mg/L for isolates that had reduced *oprD* expression.

6 Regulatory gene studies

The *mexT* gene in these 28 isolates was amplified. The *mexT* gene could not be amplified in isolate no. 2389 and the second half of the *mexT* gene could not be amplified in 19 isolates, which included four isolates with reduced *oprD* expression.

The DNA sequence of the PCR products from these isolates was compared to that of the *mexT gene* (gene bank accession no. AJ007825.1) (Appendix 7).

Mutations in the *mexT* gene of the 28 isolates are listed in Table 3.10. All except non-amplifiable isolate no. 2389 possessed a Leu_{26}→Val substitution in the MexT regulatory protein. This mutation was also present in the *mexT* gene of *P. aeruginosa* ATCC 27853. A Glu_{31}→Lys substitution was found in four isolates. Val_{110}→Phe and Ser_{135}→Thr substitutions were only present in isolate no. 2860.

Chapter 4-Discussion

1 Epidemiological characteristics of imipenem-resistant *P. aeruginosa*

Prevalence of *P. aeruginosa*

P. aeruginosa has been one of the most common organism causing nosocomial infections in many parts of the world (Lowbury *et al.*, 1970; Koneman *et al.*, 1997). An unpublished surveillance study conducted in 2005 in a regional hospital in Hong Kong showed that *P. aeruginosa* was the third most common organism isolated from hospitalized patients and was the second most common bacterial species isolated from respiratory specimens (19%). It was also one of the ten most common bacterial species isolated from blood cultures (3%) and urines (5%).

Septicemia, nosocomial pneumonia, and urinary tract and wound infections are the most common diseases caused by *P. aeruginosa* (Koneman *et al.*, 1997). Therefore, the organism is usually isolated from blood and specimens from the respiratory and urinary tract. In this study, ≥60% of the 140 isolates that were resistant to imipenem were isolated from the respiratory tract while ≥10% were from urines and wound swabs. However, none was isolated from blood cultures. This probably indicated that *P. aeruginosa* causing septicemia was usually susceptible to imipenem. However, since isolates from only four years were studied, continuous surveillance should be carried out in order to prove this hypothesis.

Most of our patients infected by imipenem-resistant *P. aeruginosa* were elderly since >60% were aged 61 or older. Our finding was similar to the surveillance study of clinical isolates of imipenem-resistant *P. aeruginosa* conducted by workers in the United States during 2001-2003. There was a preponderance of patients older than 60 years

infected by the organism (Karlowsky *et al.*, 2005). Extremes of age have been found to be a significant risk factor for nosocomial infections (Hatchette *et al.*, 2000). This is attributed to the elderly being more likely to have underlying illness, immune dysfunction and loss of the body's normal protective functions, such as skin integrity. They are usually hospitalized for a long duration (Hatchette *et al.*, 2000). These elderly patients might continuously receive imipenem or many different classes of antimicrobials. The use of several classes of antimicrobials has been reported to be associated with imipenem-resistant *P. aeruginosa*. A previous study showed that total antimicrobial use, rather than use of any specific agent, increased this risk (Muder *et al.*, 1997). A clear relationship between imipenem use and the acquisition of imipenem-resistant *P. aeruginosa* has also been proposed by other workers (Cailleaux *et al.*, 1997; Troillet *et al.*, 1997). It is interesting to note that there were more male than female patients (1:0.43). This phenomenon has also been reported previously in North America by Karlowsky *et al.* (2005) who found the ratio to be 1:0.67. This may be due to the fact that the bacteria can cause a variety of respiratory tract infections, which have a stronger association with smoking. Smoking among the elderly is much more common in males than females In Hong Kong since Lam *et al* (2007) reported that about 3635 out of 5067 (71.7%) elderly smokers are males.

2. Antibiotic susceptibilities of imipenem-resistant *P. aeruginosa*

The emergence of multi-drug resistant *P. aeruginosa*, a worldwide problem, complicates and limits therapeutic choice (Livermore and Woodford, 2000). Almost all surveillance studies reveal that there is no single drug active against all *P. aeruginosa* clinical isolates (Jones *et al.*, 2002). The problem varies in different countries. In general,

the highest rates are observed in North America and the Asia Pacific region, while the lowest rates are observed in Latin America and Europe (Rossolini and Mantengoli, 2005). For example, in Bulgaria, 49.8% of *P. aeruginosa* isolates were reported as multidrug resistant during 2001 to 2006 (Strateva *et al.,* 2007). However, in Malaysia and the United States, 5.7% and 5%, respectively of *P. aeruginosa* isolates are multiply resistant (Landman *et al.*, 2007; Raja and Singh, 2007).

Comparing to many parts of the word, the antibiotic susceptibility rate of *P. aeruginosa* was high in Hong Kong. An unpublished surveillance study from a regional hospital in Hong Kong showed that in 2005, antipseudomonal drugs such as piperacillin/tazobactam, ceftazidime, gentamicin, amikacin, ciprofloxacin and imipenem were active against >95% of the *P. aeruginosa* strains isolated. These figures were higher than those reported from an international antimicrobial surveillance program (74-95%), which included *P. aeruginosa* isolates from the United States, Canada, South America and Europe (Pfaller *et al.,* 1998). However, other than amikacin and imipenem, they are much less active against strains isolated from patients in the intensive care unit, inhibiting only 62%-76% of strains.

The prevalence of imipenem-resistant *P. aeruginosa* isolated in Hong Kong was low (3%) (infection control team of PWH, personal communication) as compared to that in Western countries. It ranges from 28% in Italy to 18% in Canada during 2000-2002 (Jones *et al.,* 2004). Studies from Korea and China also show a higher prevalence of imipenem-resistant *P. aeruginosa* than in Hong Kong, the figures being 24% and 38%, respectively (Wang *et al.,* 2003; Lee *et al.,* 2006).

All our imipenem-resistant *P. aeruginosa* isolates were also resistant to the other 11 β-lactams tested with two being resistant to all these β-lactams. The most common resistance pattern of the strains was resistance to imipenem, cefotaxime, cefoxitin and

ceftriazone (14.3%). Other than this pattern, a variety of resistance patterns (71 in total) was seen. More than 50% were resistant to the other carbapenem, meropenem and the antipseudomonal cephalosporin and ceftazidime. These figures were similar to those reported in other places such as Italy, Brazil and Japan (Kimura *et al.*, 2005; Kokis *et al.*, 2005; Toleman *et al.*, 2005) although lower than those in Brazil where >70% of *P. aeruginosa* isolates were resistant to these β-lactams (Kokis *et al.*, 2005). It is not surprising to find that most imipenem-resistant *P. aeruginosa* strains were also resistant to other β-lactams as carbapenemases class A, B and D, the β-lactamases that hydrolyse carbapenems, also hydrolyse cephalosporins including the extended-spectrum cephalosporins (Poirel and Nordmann, 2002).

Although there were 140 strains of *P. aeruginosa* in this study that were regarded as imipenem-resistant, 79% could be inhibited by 8 mg/L or 16 mg/L of imipenem, indicating the degree of resistance was intermediate. Only three isolates were inhibited by much higher concentrations of imipenem (64 mg/L or 128 mg/L). Two isolates were resistant to 17 out of the 19 antibiotics tested and one was resistant to all the 19 antibiotics. Although the number of such multiply-resistant strains was at present low, continuous surveillance of antibiotic resistance in *P. aeruginosa* should be carried out to monitor its development and to devise measures to halt its spread.

It is not surprising to find an organism to be simultaneously resistant to antibiotics with different modes of action. Although the fact that resistance to aminoglycosides, fluoroquinolones and β-lactams is mechanistically independent, cross resistance to β-lactams, aminoglycosides and fluoroquinolones is commonly reported (Rossolini and Mantengoli, 2005). In an antimicrobial surveillance program conducted by workers in Italy, 19 out of 21 of *P. aeruginosa* isolates (90%) with imipenem MICs >256 mg/L were also resistant to all of the eight tested antibiotics, including β-lactams (meropenem,

ceftazidime, cefepime, aztreonam) and aminoglycosides (gentamicin, tobramycin and amikacin) (Toleman *et al.*, 2005). In a hospital in Greece, six isolates of *P. aeruginosa* that were resistant to high levels of imipenem (MICs ≥128 mg/L) were also resistant to high levels of β-lactams (meropenem, ceftazidime, cefepime and piperacillin-tazobactam), aminoglycosides (gentamicin and amikacin) and the fluoroquinolone ciprofloxacin (Tsakris *et al.*, 2000). A possible explanation for this phenomenon is the overexpression of efflux pump systems in these imipenem-resistant isolates. The MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM pump systems can extrude a wide variety of antimicrobial agents, including quinolones, aminolycosides and most β-lactams (Kohler *et al.*, 1997; Masuda *et al.*, 2000a; Masuda *et al.*, 2000b).

Some of the aminoglycosides, especially tobramycin, and fluoroquinolones, especially levofloxacin, were still active against imipenem-resistant strains. As these drugs were active against ≥80% of our strains, they could be used to treat infections caused by imipenem-resistant *P. aeruginosa*.

3 Mechanisms of imipenem resistance in *P. aeruginosa*

3.1 Production of β-lactamases

Since the most common mechanism of β-lactam resistance is the production of β-lactamases and class B metalloenzymes (MBL) are the most common β-lactamases that hydrolyse carbapenems, the so-called carbapenemases (Livermore 1992; Livermore 2000), our investigation began with a rapid method to detect production of MBL by our strains. This method was the imipenem-EDTA disk method reported by Yong *et al.* (2002) that is able to detect MBL-producing pseudomonads with a sensitivity and specificity of 96% and 91%, respectively. The principle of the method is to use metal chelators such as

EDTA to remove zinc that is required by MBLs for their catalytic activity. Thus, in the presence of EDTA, MBL-producing strains will be susceptible to imipenem. This character is specific for MBL but not for class A and D β-lactamase (Livermore and Woodford, 2000). In this study, 20 out of the 140 strains tested showed an increase in the inhibition zone diameter of ≥8 mm around the imipenem-EDTA disk, an indication of presence of MBL (Yong *et al.*, 2002). However, no PCR product could be obtained after amplification of DNA from all these 20 strains using primers specific for MBL genes bla_{IMP}, bla_{VIM} and bla_{SIM}, indicating absence of these genes in the strains. One reason for this was that a MBL other than IMP-, VIM- and SIM-type was produced by the strains. We used primers specific for genes coding for these MBLs since the IMP- and VIM-type MBLs are the most common MBLs reported (Walsh *et al.*, 2005) while the SIM-type enzyme is first reported by Lee *et al.* (2003) from *A. baumannii* in Korea. We did not use primers specific for genes coding for the SPM- and GIM-type enzymes since these are uncommon MBLs (Walsh *et al.*, 2005). Whether our strains produced the SPM- and GIM-type enzymes or novel MBLs requires further investigation.

MBL encoding genes are usually borne on plasmids so that MBL-producing organisms can spread very rapidly especially in hospitals causing outbreaks (Senda *et al.*, 1996a; Pagani *et al.*, 2005). A study in a tertiary-care hospital in Korea shows that 17% (211 out of 1,234) of the imipenem-resistant Gram-negative isolates produce MBLs (Lee *et al.*, 2003b) and in Japan, 15% (11 out of 89) of the imipenem-resistant *P. aeruginosa* isolates produce MBLs (Kimura *et al.*, 2005). Since 14.3% of our imipenem-resistant *P. aeruginosa* isolates presumably produced MBLs, we should continue to monitor the imipenem susceptibility and production of MBLs of our isolates to prevent and control the spread of such resistant strains.

Of the 140 isolates that were screened for the production of β-lactamases by nitrocefin, only 125 were positive. Fifty-four of the β-lactamase-producing strains were shown by IEF to produce one β-lactamase while 22 produced two to four β-lactamases of pIs ranging from 7.6 to 8.2. There are a number of common plasmid-mediated β-lactamases with pIs of 7.6 - 8.2. For example, SHV-2, SHV-26, OXA-19 and CTX-M-8 enzymes have pIs of 7.6, SHV-4, SHV-18 and CTX-M-44 enzymes have pIs of 7.8 and SHV-5, SHV-9, SHV-22 and CTX-M-13 enzymes have pIs of 8.2 (http://www.lahey.org/studies). In addition, the chromosomal AmpC enzymes of *P. aeruginosa* also have pIs of 7.6, 8.1 and 8.2 (Walther-Rasmussen *et al.*, 1999). Since β-lactamases may be heterogeneous in their pI, pI value cannot, therefore, be used as a single parameter to determine the type of β-lactamases. The results of IEF are therefore compared with the results of PCR detection of β-lactamase genes.

Only two out of the 140 imipenem-resistant strains produced positive results in the detection of bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{TEM}, bla_{CTX}, bla_{SHV}, and bla_{OXA} β-lactamase genes by PCR. One isolate was shown to harbour the bla_{OXA-3} gene by DNA sequencing of the amplicon obtained after PCR using primers specific for this gene. A β-lactamase of pI 7.6 instead of 7.1, the pI value of OXA-3 β-lactamase (http://www.lahey.org/studies), was produced by this isolate. Whether the β-lactamase with a pI of 7.6 was an AmpC enzyme, OXA-19, SHV-1, SHV-2, SHV-2A, SHV-6 OR SHV-7, or even OXA-5 (pI= 7.62), OXA-7 (pI= 7.65) or OXA-6 (pI= 7.68) instead of an OXA-3 enzyme requires further investigation. The other isolate was shown to harbour the bla_{OXA-10} gene but the β-lactamase produced failed to show a band on the IEF gel. PCR is only able to detect the presence of β-lactamase genes but not the expression level of the corresponding protein. The expression level of OXA-3 and OXA-10 enzymes produced by the strains might be

not high enough to be detected by the IEF. The bla_{OXA-3} and bla_{OXA-10} are genes encoding class D extended spectrum β-lactamases (Bonnet, 2004). These are interesting findings as OXA-type β-lactamases are commonly found in *P aeruginosa* while they are rare in our isolates. Also, TEM-type, SHV-type, CTX-M-type and OXA-type enzyme, were produced by only two of them. The resistance to the extended-spectrum β-lactams in these imipenem-resistant strains was probably due to other mechanisms.

It has long been recognized that AmpC β-lactamase is produced at low levels in *P. aeruginosa* and its production is induced by certain β-lactams particularly cefoxitin and imipenem. Such induction of AmpC β-lactamase production will lead to resistance to all penicillins and cephalosporins. Imipenem is considerably stable to AmpC hydrolysis and hyperproduction of AmpC enzyme is always found to interplay with other mechanisms, such as loss of porin and overexpression of efflux pump system to cause imipenem resistance (Giwercman, *et al.*, 1990; Wu and Livermore, 1990). Hyperproduction of AmpC β-lactamase due to mutation in the regulatory gene *ampD*, *ampG* and *ampR* is commonly detected in imipenem-resistant *P. aeruginosa* (Lindberg, *et al.*, 1987; Hanson and Sanders, 1999; Kuga *et al.*, 2000). AmpC enzyme, OXA-19, SHV-6 and SHV-7 are not inhibited by clavulanic acid (Bush *et al.*, 1995). The MICs of ticarcillin in the presence of clavulanic acid for our strains that produced β-lactamases of pIs 7.6-8.2 were comparable to those of ticarcillin in the absence of clavulanic acid. Whether these strains overproduced AmpC and whether they overexpressed the *oprD* gene and together with other mechanisms resulted in imipenem resistance will be discussed later.

β-Lactamases from all the 140 imipenem-resistant strains tested were shown by a bioassay method to be unable to hydrolyze imipenem. It has been shown that GIM-1, a MBL, has a catalytic efficiency that is much lower than that of other MBLs such as IMP-

1, VIM-1, VIM-2, and SPM-1 (Laraki *et al.*, 1999; Docquier *et al.*, 2003; Murphy *et al.*, 2003; Castanheira *et al.*, 2004). Thus, whether our strains produced MBLs with weak imipenem hydrolytic activity that could not be detected by the bioassay that we used should be investigated further by β-lactamase kinetics studies (Lorian, 1995).

3.2 Outer membrane permeability

It has been shown that the outer membrane protein OprD is specific for the entry of carbapenems into bacterial cells and imipenem resistance is usually associated with OprD loss (Livermore, 1992; Huang and Hancock, 1993; Kohler *et al.*, 1999). In this study, SDS-PAGE of outer membrane proteins extracted from 28 tested isolates showed that a ~46 kDa protein was present in only 20 isolates. Nine out of these 28 isolates were shown to have reduced *oprD* expression by real-time RT-PCR since their normalized expression relative to *P. aeruginosa* ATCC 27853 were less than 1. One of these isolates had an imipenem MIC of 128 mg/L and two with 64 mg/L. The mode and median MIC of imipenem were 32 mg/L for isolates with reduced *oprD* expression while they were 16 mg/L for isolates had reduced *oprD* expression. These findings suggest that loss of outer membrane protein OprD occurred more often in strains with high resistance to imipenem than strains with low to intermediate resistance to imipenem in our study.

Nineteen isolates showed an increase in *oprD* gene expression. However, the 46 kDa OprD-like protein was absent in two isolates although their *oprD* expression was 4.86 and 1.07 fold respectively. This was probably due to a disruption in translation from mRNA to tRNA and resulting in absence of the OprD protein (Koneman *et al.*, 1997). In contrast, the *oprD* expression was 0.49-fold for one isolate but a band corresponding to that of the OprD protein was visible on the gel after SDS-PAGE. However, in most of the

previous studies reported, an OprD-like protein is not detected on the SDS-PAGE gel for the strains with decreased expression of *oprD* gene (Quale *et al.*, 2006).

Thus, OprD loss due to reduced *oprD* expression in the nine isolates was probably responsible for imipenem resistance. However, this might not be the sole mechanism. Firstly, the OprD protein is a specific channel for imipenem but not other β-lactams, including meropenem (Huang and Hancock, 1993). Studies have shown that organisms which lost the OprD had an MIC of meropenem four-fold lower than that of imipenem (Livermore, 1992; Kohler *et al.*, 1999). However, only two of our nine isolates had MICs of meropenem four-fold lower than that of imipenem while the rest had MICs of meropenem that were either the same (in one isolate) or ≥2-fold higher than those of imipenem. In addition, five of these nine isolates were resistant to four to 11 β-lactams other than imipenem while four were resistant to 12. Secondly, strains with loss of OprD but without other resistance mechanisms usually only exhibited a relatively low-level resistance (4 mg/L to 16 mg/L) to imipenem (Pai *et al.*, 2001).

Whether overproduction of AmpC enzyme contributed to imipenem resistance in these strains requires further investigation. Pai *et al.* (2001) showed that strains with loss of the OprD protein as well as AmpC overproduction are resistant to imipenem and other β-lactams.

3.3 Effects of regulatory gene mutations

All 28 isolates that were selected for further investigation of membrane permeability were shown to possess a Leu_{26}→Val substitution in the MexT regulatory protein. However, this substitution probably does not affect MexT function (Maseda *et al.*, 2000). Three other substitutions, namely Glu_{31}→Lys, Val_{110}→Phe and Ser_{135}→Thr

detected in five isolates have not been reported previously. The role of these substitutions in affecting the function of the MexT protein requires further investigation.

The *mexT* gene could not be amplified in one isolate, and the second half of the *mexT* gene could not be amplified in 19 isolates, suggesting major mutations were present in the non-amplifiable region. Besides, *oprD* expression was reduced in four of the isolates. The *mexT* gene is a negative regulator for the expression of *oprD* and mutations can convert it from nonfunctional to functional (Kohler *et al.*, 1999; Ochs *et al.*, 1999; Maseda, *et al.*, 2004). However, some of the isolates with a nonfunctional *mexT* gene had down-regulated the expression of *oprD*, suggesting that other factors were also involved. This finding has also been reported by Quale *et al.* (2006). In another study, a newly described regulator *mexS* has been proposed to function as a putative negative regulator for OprD protein (Kohler *et al.*, 1999; Maseda *et al.*, 2000b).

The *mexT* gene, on the other hand, is reported as the positive regulator of the MexEF-OprN system which is responsible for the export of antibiotics such as fluoroquinolones, trimethoprim and chloramphenicol (Kohler *et al.*, 1997; Kohler *et al.*, 1999; Maseda *et al.*, 2000b). The *mexEF-oprN* is overexpressed in the *nfx-C*-type mutants of *P. aeruginosa*, which are resistant to quinolones, imipenem, and choramphenicol (Kohler *et al.*, 1997). The *nfxC*-type mutants are also cross-resistant to imipenem since the isolates also have decreased expression of OprD (Aubert *et al.*, 1992; Fukuda *et al.*, 1995; Masuda *et al.*, 1995). The MexEF-OprN system was suspected to be overexpressed in nine isolates with a functional *mexT* gene. However, the correlation between overexpression of MexEF-OprN and decreased expression of OprD protein could not be drawn since other factors could be involved in regulating the *oprD* gene in the isolates.

4 Integrons in imipenem-resistant *P. aeruginosa*

A class 1 integron was detected in >60% of our imipenem-resistant isolates. This integron is also commonly found in imipenem-resistant *P. aeruginosa* isolates around the world (Walsh *et al.*, 2005). Genes coding for imipenem-hydrolyzing β-lactamases including IMP- and VIM-type have been reported to be present on class 1 integrons (Walsh *et al.*, 2005). It is not surprising that we did not detect class 2 integrons in any of our isolates studied since they are much more uncommon than class 1 integrons (Hall and Collis, 1995).

Gene cassettes were detected in the majority of our integron-positive isolates (79%). The non-amplifiable gene cassettes in the isolates are probably due to the loss of 3'-conserved segments (*qacEΔ1* and *sulI*). Absence of the 3'-conserved segment has been reported (Naas *et al.*, 1998; Rosser and Young, 1999). It is interesting to note that all isolates shown to produce a MBL by the imipenem-EDTA disk method were positive for class 1 integron but 16 had lost the 3' conserved region. However, the 3' conserved region was detected in 95% of those isolates that were integron-positive and shown not to produce MBL by the same method. The difference between the two was statistically significant ($p<0.05$). It is possible that the majority of these MBL-producing strains had a major mutation in the 3' conserved region.

No resistance genes were detected in the gene cassettes of >50% of the integron-positive isolates. The bla_{OXA-3} and bla_{OXA-10} genes, present in two of the isolates, were present on gene cassettes of a class 1 integron. The *aadA1*, *aadA2* and *aadA6* genes, detected in some of our integron-positive isolates, code for adenylytransferases that lead to resistance to streptomycin and spectinomycin (Shaw *et al.*, 1993). Since we did not test the susceptibility of our isolates to these two aminoglycosides that are infrequently used for treatment of *P. aeurginosa* infections, the effect of the presence of these genes on

aminoglycoside susceptibility could not be determined. However, it is not surprising to find these *aadA* genes in gene cassettes in our isolates since they have been reported previously (Hall and Collis, 1995; Recchia and Hall, 1995). Other than these, no other resistance genes were detected in our isolates.

5 Conclusions

Imipenem-resistant *P. aerugniosa* strains were commonly isolated from specimens from the respiratory tract, urinary tract and wounds of patients who were mainly elderly males.

Of the 140 imipenem-resistant *P. aeruginosa* isolates studied, >50% were resistant to ten to 19 antibiotics. The majority produced a variety of β-lactamases while one each produced the OXA-3 and OXA-10 enzyme. Twenty were shown by a bioassay to produce MBL but common MBL genes including bla_{IMP}, bla_{VIM}, bla_{SIM} were not detected in them. Crude β-lactamase extracts from these isolates were unable to hydrolyze imipenem. Reduced expression of the *oprD* gene was detected in nine out of the 28 isolates that were selected for further study of the mechanisms of imipenem-resistance. Although mutations that converted *mexT* regulator gene from nonfunctional to functional were detected in strains with decreased expression of the *oprD* gene, other factors were probably also responsible in regulating the expression of this gene.

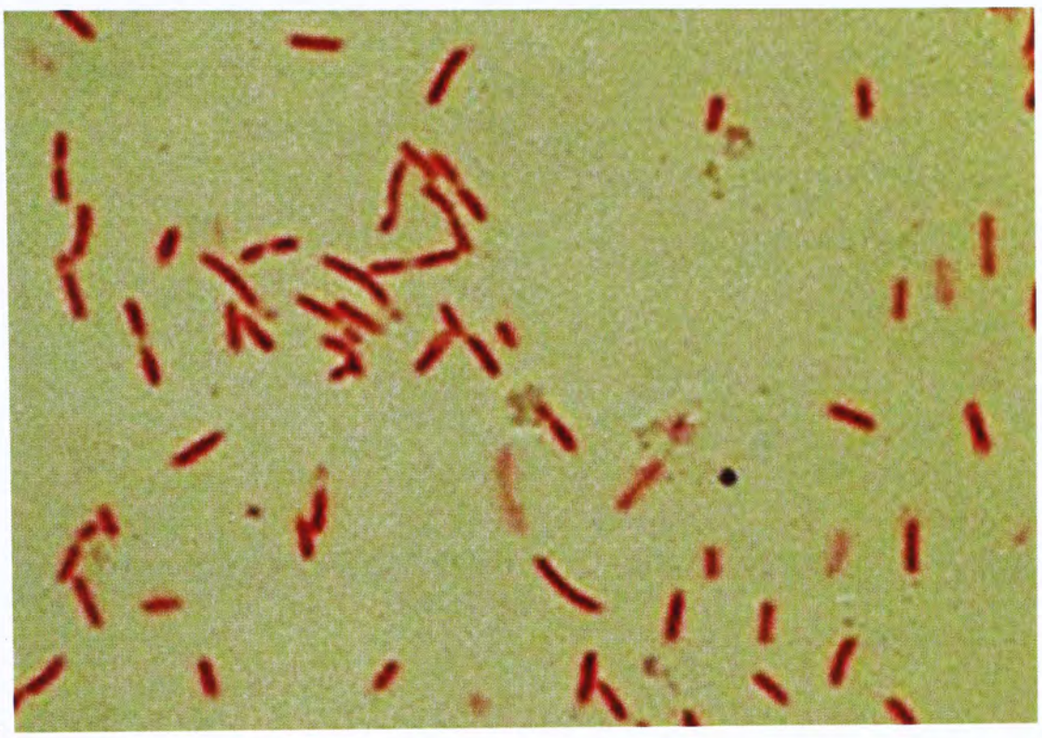
A class 1 integron was detected in the majority of isolates. The bla_{OXA-3}, bla_{OXA-10}, *aadA1, aadA2* and *aadA6* genes were present in gene cassettes of the integrons. The 3' conserved region could be absent in isolates that produced a MBL.

6 Areas for further study

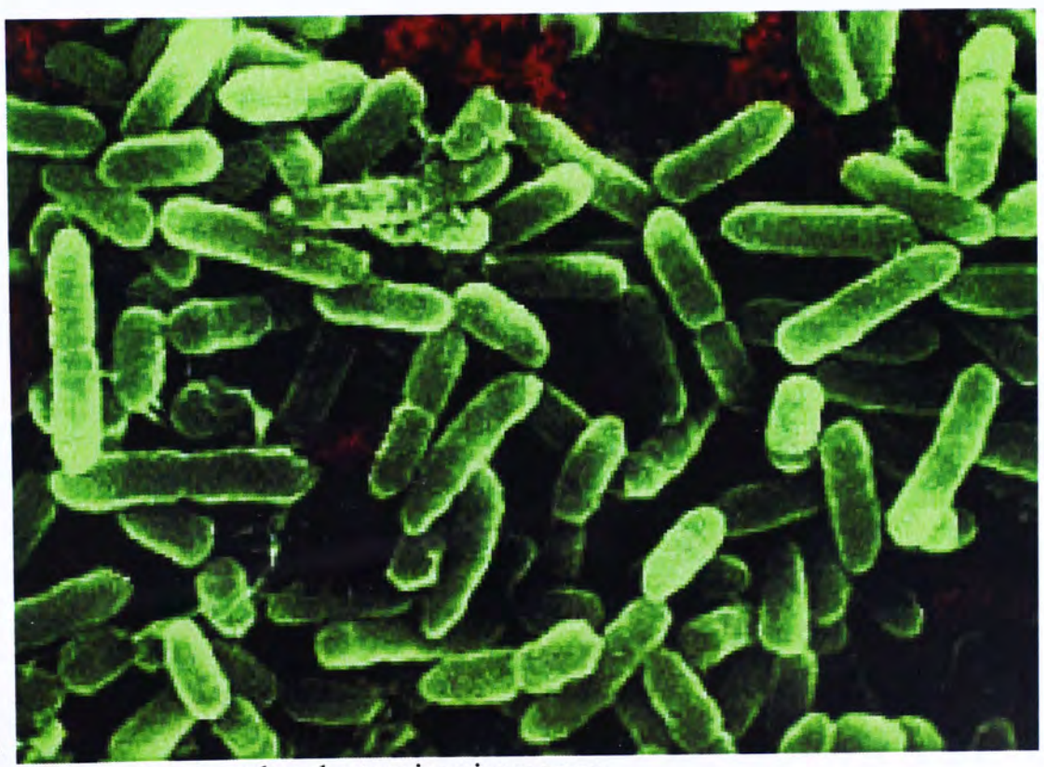
Although results from the present study have elucidated some of the mechanisms of imipenem-resistance in our *P. aeurginosa* isolates, several questions remain unanswered and investigations to elucidate them are worth pursuing:

1. Production of carbapenemases is one of the mechanisms of imipenem resistance, however, it was detected in only a minority of our isolates. Besides, we were unable to characterize the carbapenemases other than to detect them using a phenotypic method. Whether these isolates produced novel MBL(s) should be investigated by kinetic studies and the encoding genes by molecular cloning.
2. The AmpC enzyme is proposed to be overproduced in our strains. Whether overexpression of AmpC is responsible for imipenem-resistance should be studied by characterizing mutations leading to AmpC hyperproduction.
3. Most of our imipenem resistant strains were simultaneously resistant to other classes of antibiotics. We have shown that the OprD protein was absent in some of these isolates, thus leading to imipenem-resistance. Whether the *mexEF-oprN* gene was also overexpressed causing these strains to be resistant to other antibiotics can be elucidated by performing real-time RT-PCR on the *mexE* gene.
4. Whether the imipenem-resistant *P. aeruginosa* strains were progenies of a single or limited number of clones should be investigated by molecular typing using methods such as pulsed-field gel electrophoresis (PFGE) or PCR.

Figure 1.1. Morphology of *P. aeruginosa*

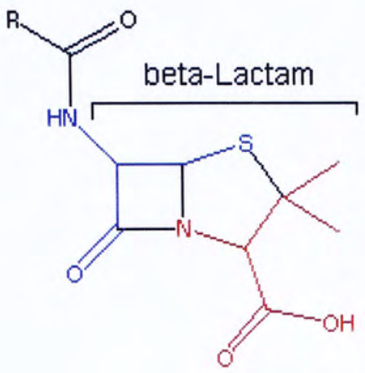


Gram-strain of *P. aeruginosa.*
©Diane P. Yolton. http://opt.pacificu.edu/ce/catalog/13036-AS/Antibiot.html. June 30, 2007.



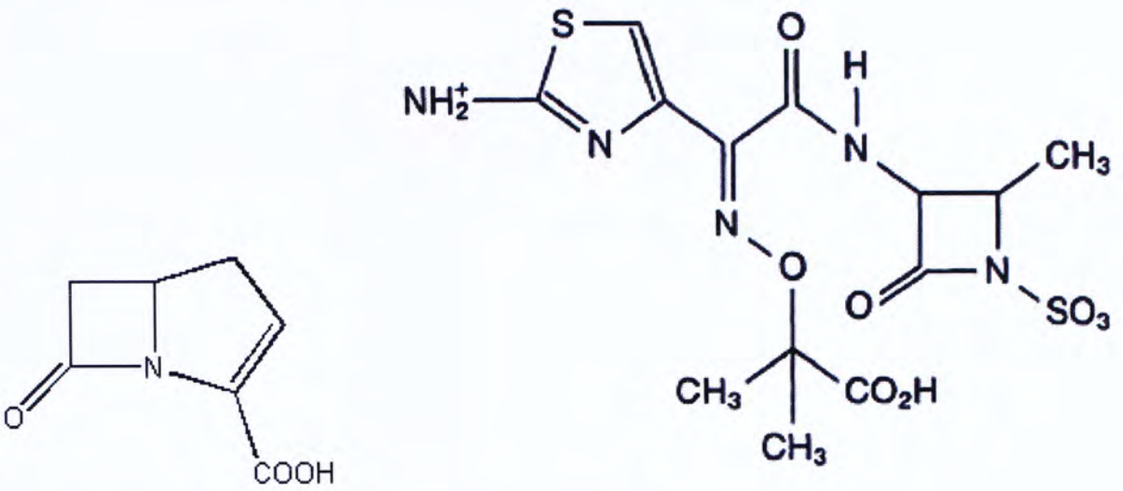
P. aeruginosa under electronic microscope
©James A Sullivan. www.cellsalive.com. June 30, 2007

Figure 1.2. Structure of β-lactam



©University of Leeds, Faculty of biological science. http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/icu8/antibiotics/wall.html. June 30, 2007

Figure 1.3. Structure of carbapenem and its derivate imipenem.



Carbapenem Imipenem

©University of Leeds, Faculty of biological science. http://www.bmb.leeds.ac.uk/mbiology/ug/ugteach/icu8/antibiotics/wall.html. June 30, 2007

Figure 1.4. Efflux pump system of Gram-negative bacteria

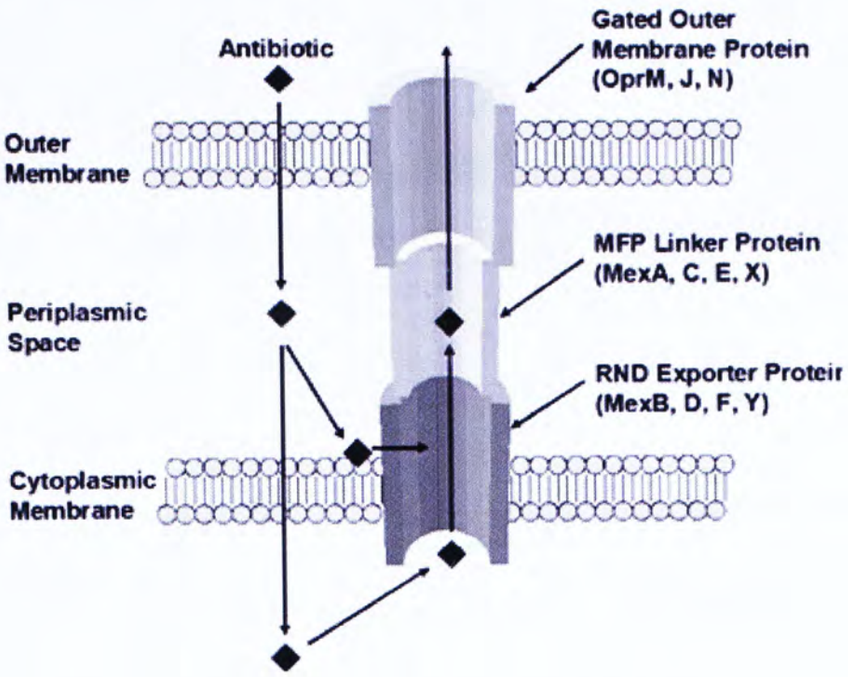


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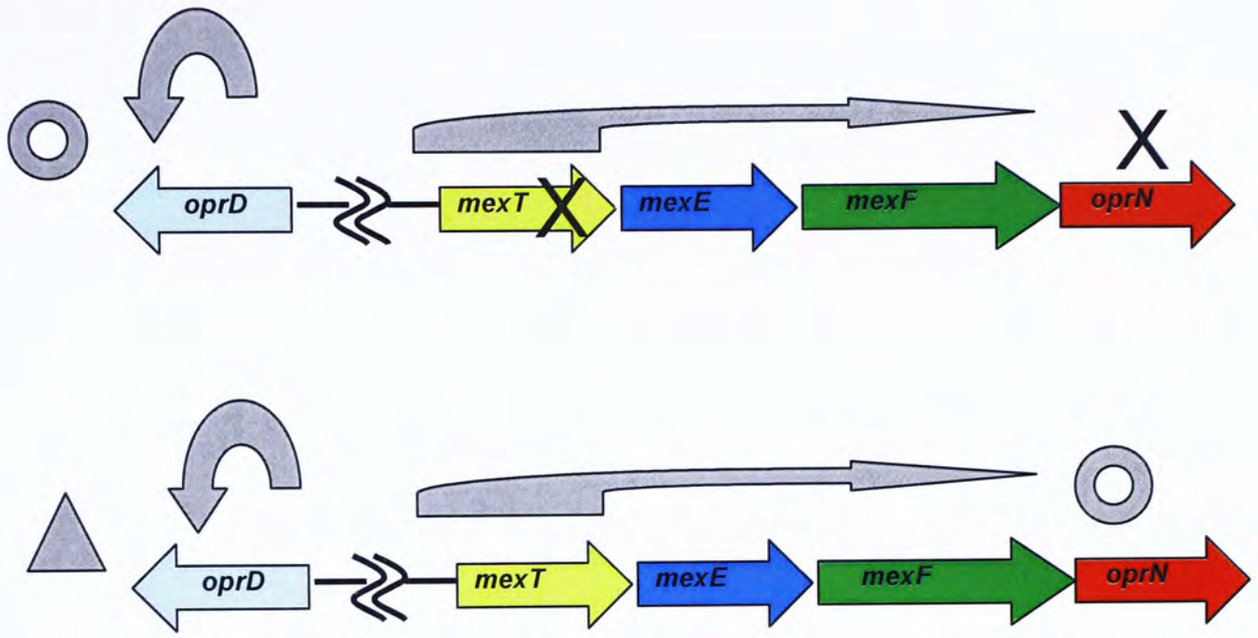
Figure 1.5. Schematic representation of expression of *mexEF-oprN* and *oprD* genes regulated by *mexT* gene



: High-level expression

: Very low-level expression

X : No expression or no function

Mutations covert *mexT* from nonfunctional to functional and lead to down-regulate the expression *oprD* and up-regulate the expression of *mexEF-oprN*.

Figure 1.6. Schematic representation of class 1 integron

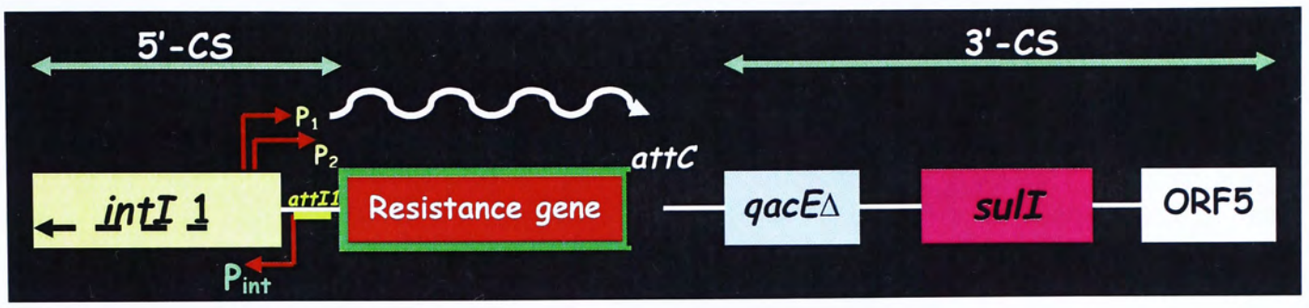
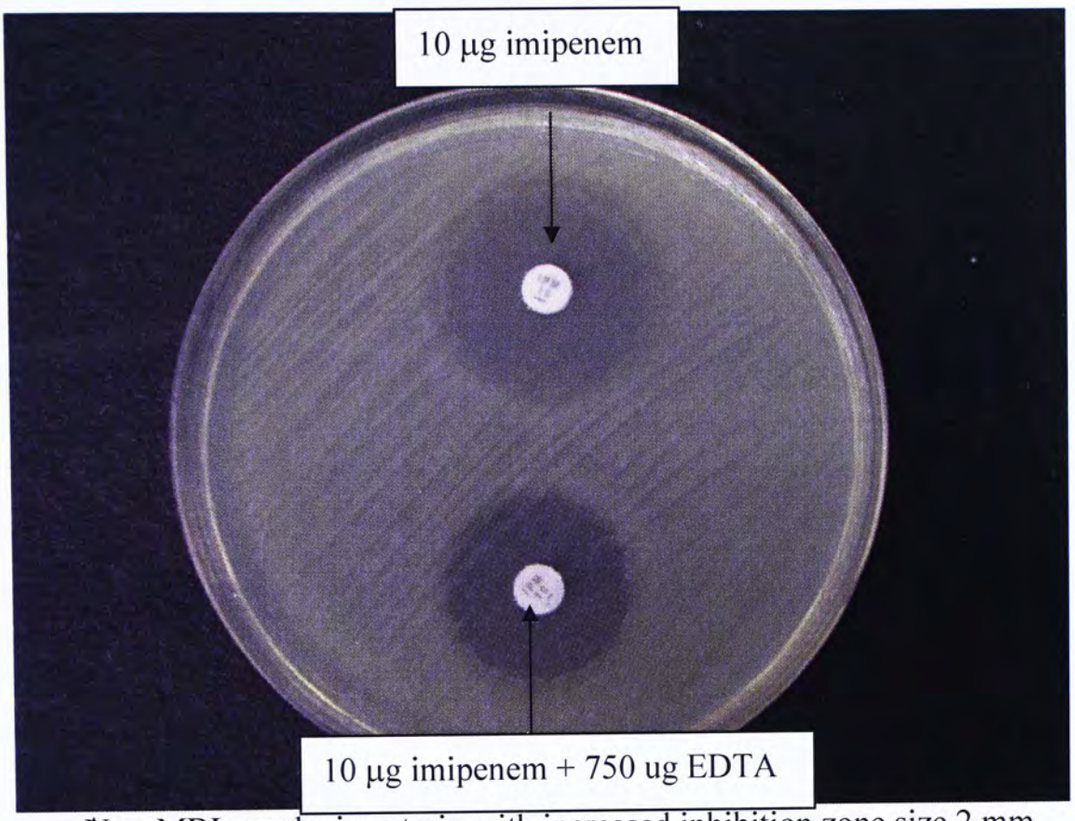
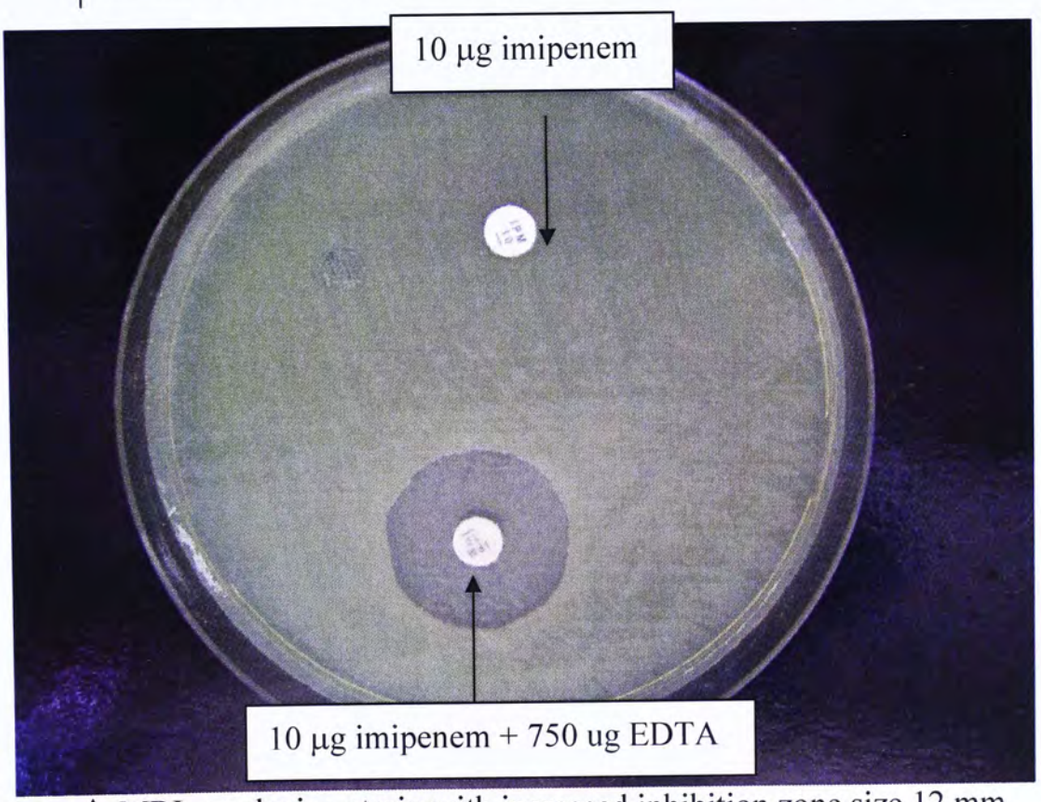


Figure 3.1. Phenotypic detection of non-MBL and MBL producing strains



Non-MBL producing strain with increased inhibition zone size 2 mm



MBL producing strain with increased inhibition zone size 12 mm

Figure 3.2. Bioassay to test the ability of β-lactamases to hydrolyse imipenem

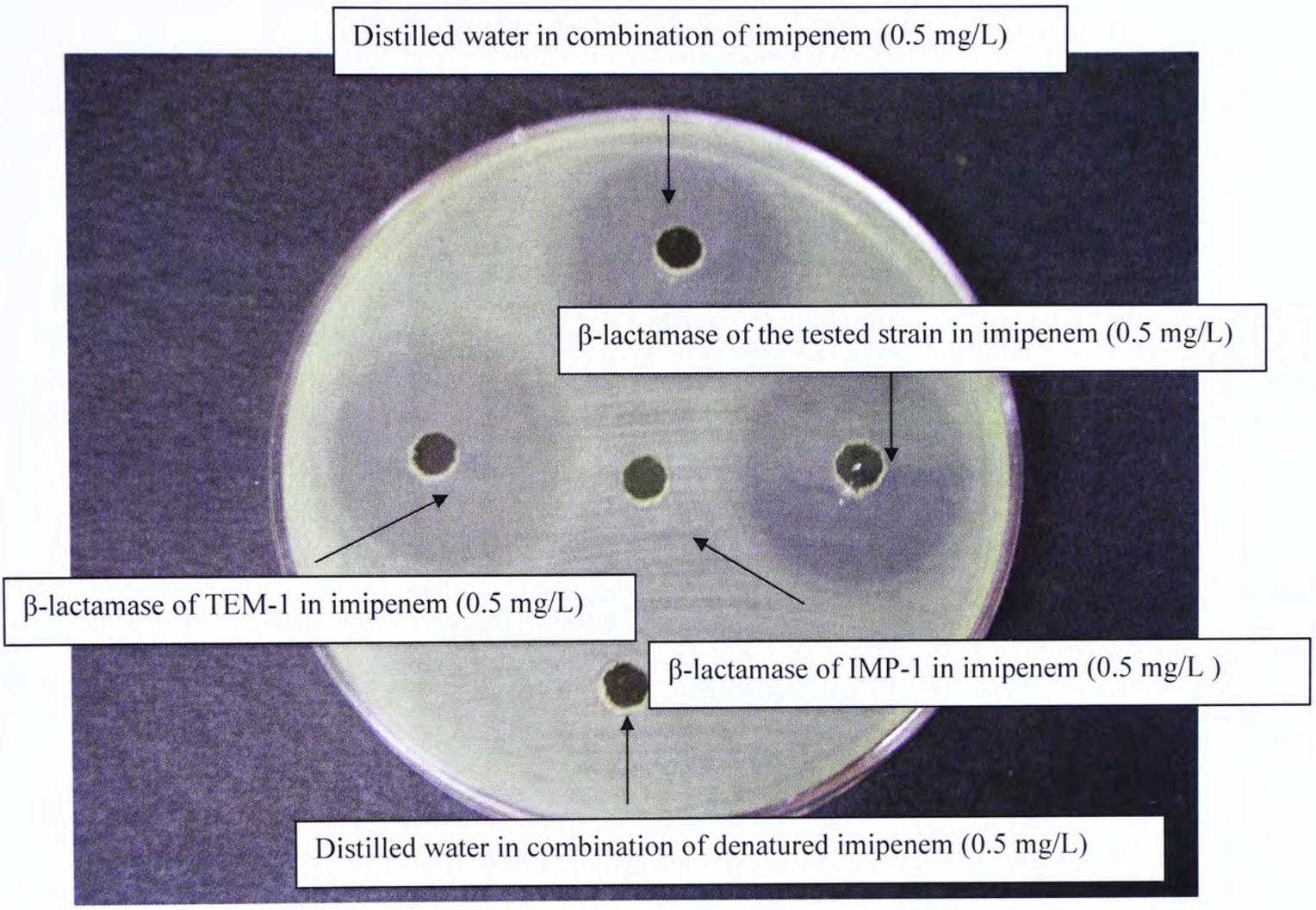
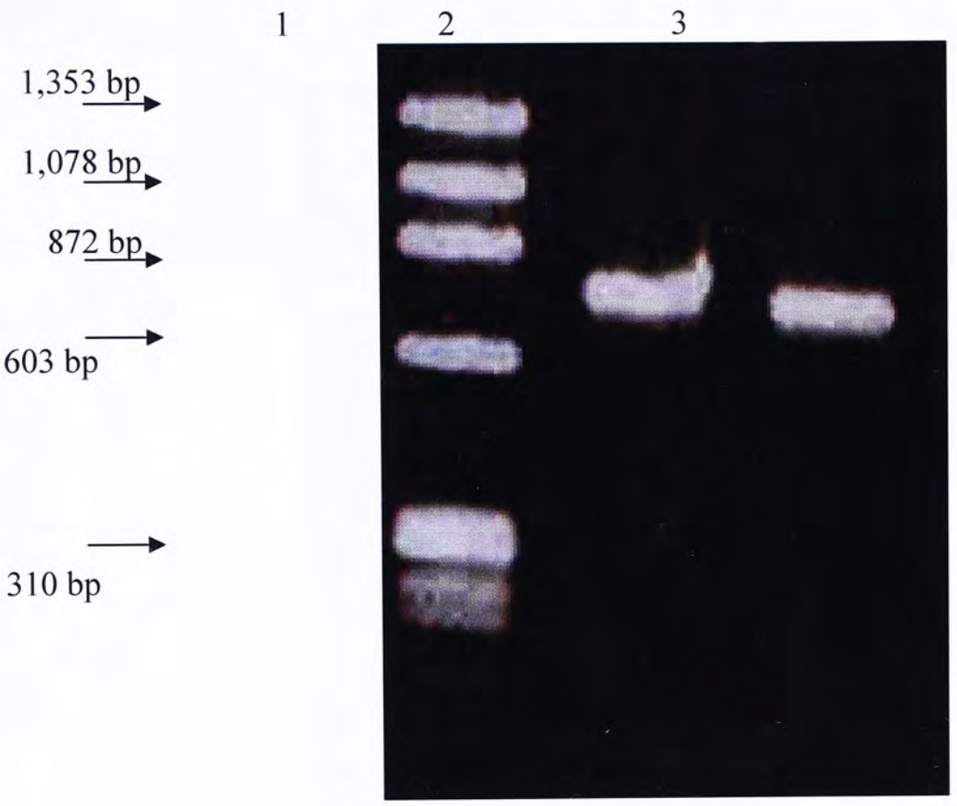


Figure 3.3. Agarose gel electrophoresis of PCR products for bla_{OXA-3} and bla_{OXA-10} obtained after amplification using primers specific for OXA group I and OXA group II

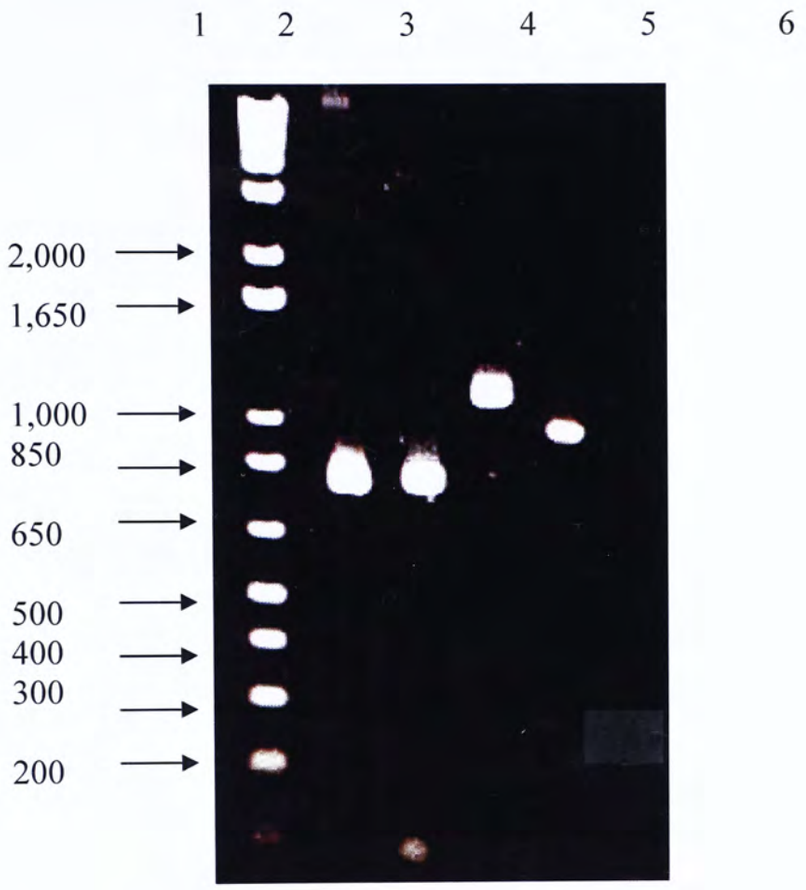


Lane 1: The size marker, ΦX174 DNA/*Hae* III marker

Lane 2: 760-amplicons of bla_{OXA-10} from isolate 2244

Lane 3: 700-amplicions of bla_{OXA-3} from isolate 4638

Figure 3.4. PCR products of *aadA1, aadA2* and *aadA6* obtained after amplification using primers for gene cassettes



Lane 1: 1 kb plus DNA ladder

Lane 2: 800-bp amplicons of *aadA1*

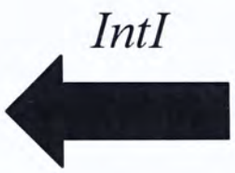
Lane 3: 800-bp amplicons of *aadA6*

Lane 4: 1,000 amplicons of *aadA2*

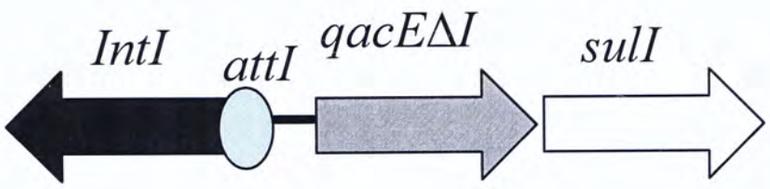
Lane 5: 900 amplicons of *aadA6*

Figure 3.5. Structure of integrons

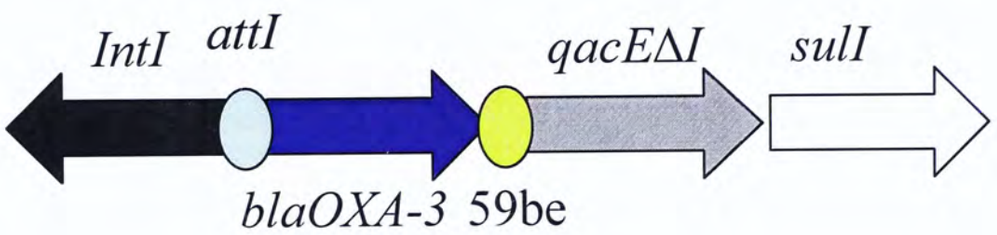
1.



2.



3.



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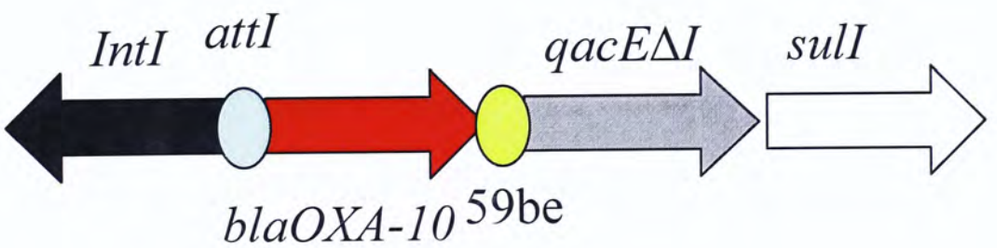
4.



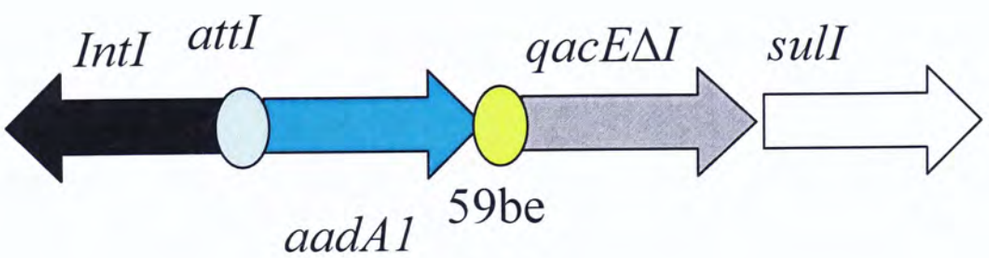
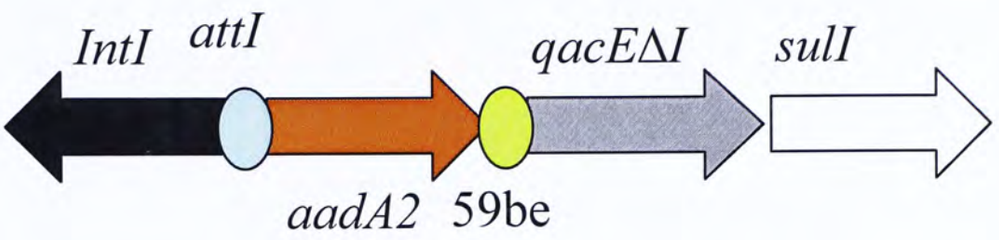
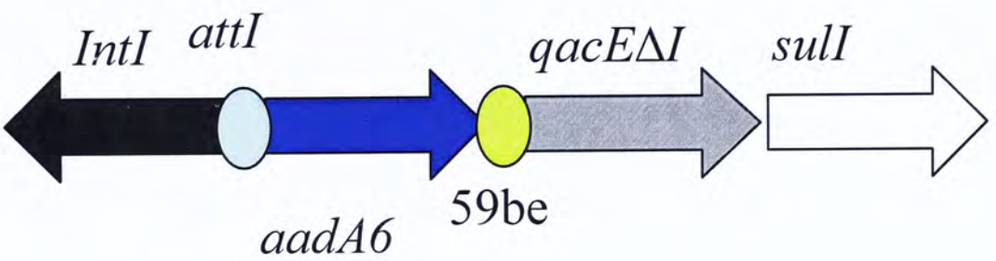


Figure 3.5 (Continued).

5.

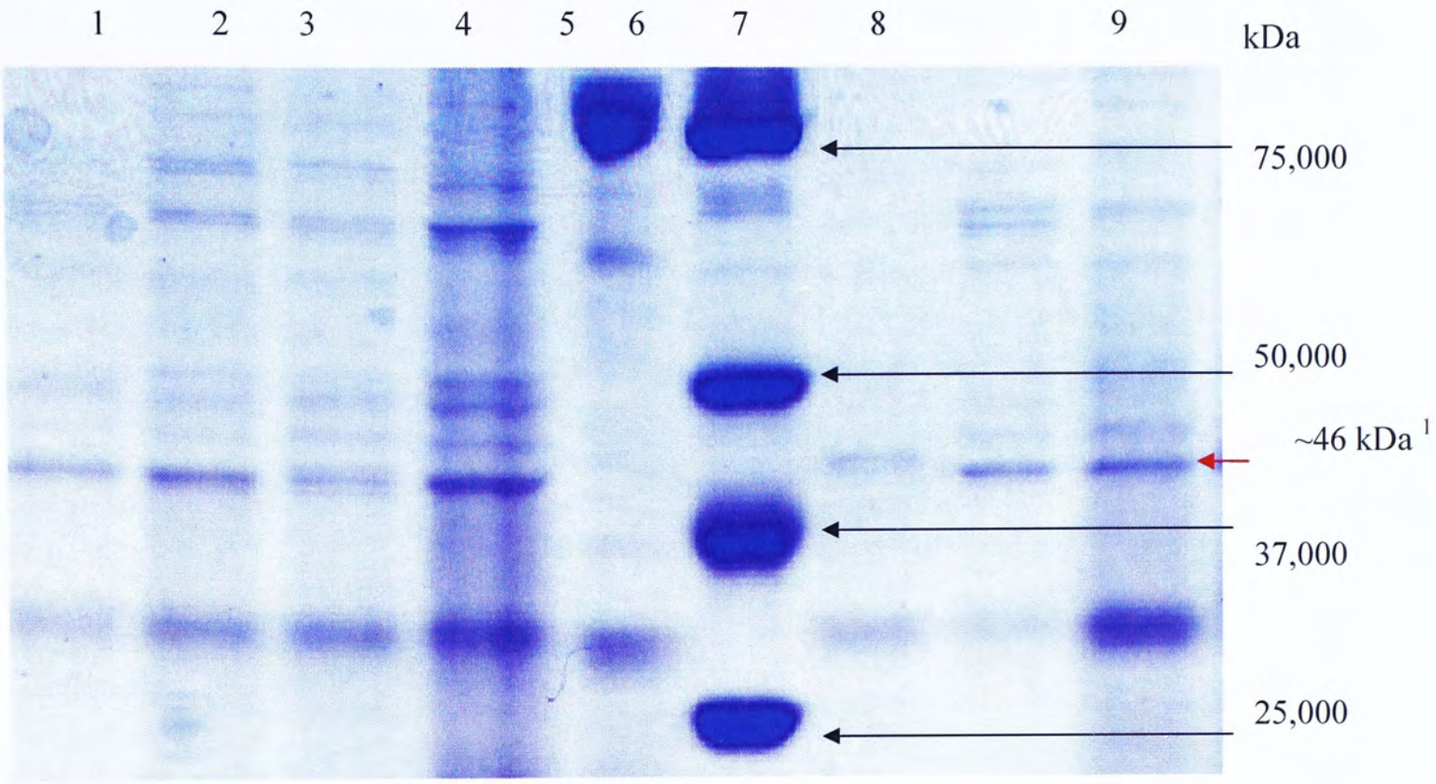


6.



(1) Class 1 integrase. (2) Class 1 integron gene cassette. (3) Class 1 integron gene cassette encoding bla_{OXA-3}. (4) Class 1 integron gene cassette encoding bla_{OXA-10}. (5) Class 1 integron gene cassette encoding *aadA1*. (6) Class 1 integron gene cassette encoding *aadA2*. (7) Class 1 integron gene cassette encoding *aadA6*.

Figure 3.6. SDS-PAGE of outer membrane proteins (OMPs)



Lane	Strain	Presence of 46 kDa
1	Isolate no. 2389	Yes
2	Isolate no. 455	Yes
3	Isolate no. 88	Yes
4	ATCC 27853	Yes
5	Isolate no. 2860	No
6	Protein markers (Prestained SDS-PAGE standards broad range)	
7	Isolate no. 1287	Yes
8	Isolate no. 4397	Yes
9	Isolate no. 1122	Yes

[1] The size of OpD protein is ~46 kDa

Table 2.1. List of materials, softwares and equipment used in this study

	Sources
Antibiotics	
Aminoglysides-	
Amikacin	Sigma, MO, USA
Gentamicin	Sigma
Netilmicin	Schering Corporation, Bloomfield, USA
Tobramycin	American Cyanamid Company, New York, USA
β-lactams-	
Piperacillin	Sigma
Tazobactam	Sigma
Ticarcillin	Sigma
Clavulanic acid	GlaxoSmithKline, Greenford, England
Cefoxitin	Sigma
Cefotaxime	Sigma
Ceftazidime	GlaxoSmithKline
Ceftriaxone	Sigma
Cefepime	Bristol-Myers Squibb Co., NY , USA
Aztreonam	Bristol-Myers Squibb Co.
Imipenem	Merck, VA, USA
Meropenem	Zeneca Pharmaceuticals Group, Wilmington, USA
Fluoroquinolones-	
Levofloxacin	Sigma
Norfloxacin	Sigma
Ofloxacin	Sigma
Antibiotics discs	
Imipenem (10 μg)	Oxoid, Hampshire, England

	Sources
Media	
Luria-Bertani (LB) broth	Difco Laboratories, MI, USA
Mueller-Hinton (MH)	Oxoid
Nutrient agar	Oxoid
Markers	
Φ174/*Hae* III digest	Amersham Pharmacia Biotech, Upsala, Sweden
SDS-PAGE molecular weight standard (low range)	Bio-Rad, CA, USA
Kits	
API20E system	BioMerieux, RCS Lyon, France
Bio-Rad BCATM Protein Assay	Bio-Rad
MicroSpin S-300 HR columns	Amersham Pharmacia Biotech

RNeasy Mini kit	Qiagen, CA, USA
TaqMan EZ RT-PCR Core Reagents kit	Applied Biosystem, New Jersey, USA
Chemical	
Acetic acid	Merck
BBL oxidase	Becton Dickson & Co., MD, USA
β-Mercaptoethanol	Merck
Bromophenol blue	BDH Chemicals Ltd., Poole, UK
Ethylenediamine tetraacetic acid (EDTA)	Amersham Pharmacia Biotech
Ethidium bromide	Sigma, Missouri, USA
Glycerol	Merck
Hydrochloric acid	Sigma
Lysozyme	Sigma
Methanol	Merck
Nitrocefin	GlaxoSmithKline
Oxidase reagent	Oxoid
Phosphoric acid	Merck
Sodium carbonate	Sigma
Sodium chloride	BDH
Sodium hydroxide	Merck
Sodium phosphate	Merck
Tris-borate	Merck
Triton-100	USB, Cleveland, USA
Gels	
Seakem®LE agarose	Amersharm Pharmacia Biotech
CriterionTM XT precast gel	Bio-Rad
Precast polyacrylamide pH 3 to 10 gel	Novel Experimental Technology, San Diego, USA
Instruments	
ABI Prism ® 7700 Sequence Detection Sytem	Applied Biosystem
Automatic inoculator MIC-2000	Dynatech, Virginia, USA
Centrikon T-2080 centrifuge	Kontron Instruments, Basel, Switzerland
Chemidoc XRS	Bio-Rad
Eppendorf centrifuge	Eppendorf AG, Hamburg, Germany
Gel documentation system	Amersham Pharmacia Biotech
GNA 100 electrophoresis apparatus	Amersham Pharmacia Biotech
Multiskan ascent	Thermo Electron Corporation, Waltham, MA, USA
Multiphor II electrophoresis unit	Amersham Pharmacia Biotech
MultiTemp II thermostatic circulator	Amersham Pharmacia Biotech
PowerPac HCTM	Bio-Rad

NanoDrop ND-1000	NanoDrop Technologies, Inc, Wilmington, DE, USA
Thermol controller (Gene Amp PCR system 9600)	Perkin Elmer, Boston, USA
Software	
Chromas 1.42 software	Brisbane, Queensland, Australia
ND-1000 3.3.0	NanoDrop Technologies
Quantity One	Bio-Rad
Sequence Detection System software version 1.7.	Applied Biosystem
SDS-PAGE reagents	
20X XT-reducing agent	Bio-Rad
4X XT-sample buffer	Bio-Rad
20X XT MOPS running buffer	Bio-Rad
Coomassie blue G-250	Bio-Rad
SDS-PAGE molecular weight standard (low range)	Bio-Rad
Real-time RT-PCR reagent	
Dnase I	Progema, Madision, USA
Recombinant RNasin® Ribonuclease inhibitor	Progema
RNase-free water	Invitrogen
RNase-free 25 mM EDTA	Progema
PCR reagents	
10X Taq Buffer	Amersham Pharmacia Biotech
DNA polymerization mix	Amersham Pharmacia Biotech
Primers	Invitrogen, CA, USA
Taq DNA polymerase	Amersham Pharmacia Biotech

Table 2.2. Solvents used for dissolving the antibiotics and concentration range of antibiotics tested

Antibiotics	Solvent	Concentration range (mg/L)
Aminoglysides-		
Amikacin	Distilled water	0.5-256
Gentamicin	Distilled water	0.06-128
Netilmicin	Distilled water	0.12-256
Tobramycin	Distilled water	0.06-128
β-lactams-		
Piperacillin	Distilled water	2-1024
Piperacillin/ tazobactam	Distilled water	2/2-1024/2
Ticarcillin	Distilled water	2-1024
Ticarcillin/ clavulanic acid	Distilled water	2/2-1024/2
Cefoxitin	Distilled water	0.5-256
Cefotaxime	Distilled water	0.25-512
Ceftazidime	Saturated sodium carbonate	0.12-256
Ceftriaxone	0.2% sodium carbonate	0.12-256
Cefepime	0.1M phosphate buffer, pH 6.0	0.06-128
Aztreonam	Saturated sodium carbonate	0.06-128
Imipenem	0.01M phosphate buffer, pH 7.0	0.06-128
Meropenem	Distilled water	0.06-128
Fluoroquinolones-		
Levofloxacin	0.1N sodium hydroxide	0.06-128
Norfloxacin	0.1N sodium hydroxide	0.06-128
Ofloxacin	0.1N sodium hydroxide	0.06-128

Table 2.3. Components of PCR for *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SIM}$, *bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{SHV}$, and *bla*$_{OXA}$

Reagent	Stock concentration	Volume (µl)	Final concentration
Forward primers	25 µM	0.5	0.5 µM
Reverse primers	25 µM	0.5	0.5 µM
dNTPs	10 mM	0.5	0.2 mM
10X *Taq* buffer	10 X	2.5	1 X
Taq DNA polymerase	1 U/µl	0.2	0.2 U
DNA template		1	-
Nuclease free water		19.55	
Total		25	

Table 2.4. Primers for bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{TEM}, bla_{CTX}, bla_{SHV}, and bla_{OXA}

Primer	Target	Oligonucleotide sequence	Product size (bp)	Reference
TEM-F	TEM-1 and derivatives	5'-ATA AAA TTC TTG AAG ACG AAA-3'	1079	Bonnet *et al.*, 1999
TEM-R		5'-GAC AGT TAC CAA TGC TTA ATC A-3'		
SHV-F	SHV-1 and derivatives	5'-TGG TTA TGC GTT ATA TTC GCC-3'	870	Kim *et al.*, 1998
SHV-R		5'-GGT TAG CGT TGC CAG TGC T-3'		
CTX-F	CTX-M-1,-2,-9 Groups	5'-CGC TTT GCG ATG TGC AG- 3'	550	Bonnet *et al.*, 2001
CTX-R		5'-ACC GCG ATA TCG TTG GT-3'		
IMP-F	IMP-1 and derivates	5'-ATG AGC AAG TTA TYW GTA TTC-3'	766	Toleman *et al.*, 2005
IMP-R		5'-GCT GCA ACG ACT TGT TAG-3'		
VIM-F	VIM-1 and derivates	5'-TTA TGG AGC AGC AAC GAT GT-3'	622	Toleman *et al.*, 2005
VIM-R		5'-CGA ATG CGC AGC ACC AGG-3'		
SIM-F	SIM-1 and derivates	5'-TAC AAG GGA TTC GGC ATC G-3'	552	Lee *et al.*, 2005
SIM-R		5'-TAA TGG CCT GTT CCC ATG TG-3'		

Table 2.4. Primers for *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SIM}$, *bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{SHV}$, and *bla*$_{OXA}$ (Continued)

Primer	Target	Oligonucleotide sequence	Product size (bp)	Reference
OXA-10F	OXA group I	5'-TCT TTC GAG TAC GGC ATT AGC-3'	760	Naas *et al.*, 2000
OXA-10R		5'-CCA ATG ATG CCC TCA CTT TCC-3'		
OXA-2F	OXA group II	5'-GCC AAA GGC ACG ATA GTT GT-3'	700	De-Champs *et al.*, 2000
OXA-2R		5'-GCG TCC GAG TTG ACT GCC GG-3'		
OXA-1F	OXA group III	5'-AGC CGT TAA AAT TAA GCC C-3'	908	Aubert *et al.*, 2001
OXA-2R		5'-CTT GAT TGA AGG GTT GGG CG-3'		

Table 2.5. Components of PCR for *intI1* and *intI2*

Reagent	Stock concentration	Volume (µl)	Final concentration
Forward primers	25 µM	0.5	0.5 µM
Reverse primers	25 µM	0.5	0.5 µM
dNTPs	10 mM	0.5	0.2 mM
10X *Taq* buffer	10 X	2.5	1 X
Taq DNA polymerase	1 U/µl	0.2	0.2 U
DNA template		1	-
Nuclease-free water		19.55	
Total		25	

Table 2.6. Primers used for *intI1* and *intI2*

Primer	Target	Oligonucleotide sequence	Product size (bp)	Reference
IntI1 F	*intI1*	5'-GCCTTGCTGTTCTTCTACGG-3'	558	Gonzalez *et al.*, 1998
IntI1 R		5'-GATGCCTGCTTGTTCTACGG-3'		
IntI2 F	*intI2*	5'-CACGGATATGCGACAAAAAGGT-3'	789	Gonzalez *et al.*, 1998
IntI2 R		5'-GTAGCAAACGAGTGACGAAATG-3'		

Table 2.7. Primers for gene cassettes and 3'-conserved segments

Primer	Target	Oligonucleotide sequence	Product size (bp)	Reference
GC F	Gene cassette	5'-GGCATCCAAGCAGCAAGC-3'	Variable	Zhang *et al.*, 2004
GC R		5'-AAGCAGACTTGACCTGAT-3'		
qacE△1-F	3'-conserved	5'-ATCGCAATAGTTGGCGAAGT-3'	798	Zhang *et al.*, 2004
sul1-B	segments	5'-GCAAGGCGGAAACCCGCGCC-3'		

Table 2.8. Components of PCR for gene cassette

Reagent	Stock concentration	Volume (µl)	Final concentration
Forward primers	25 µM	0.5	0.5 µM
Reverse primers	25 µM	0.5	0.5 µM
dNTPs	10 mM	0.5	0.2 mM
10X *Taq* buffer	10 X	2.5	1 X
Taq DNA polymerase	1 U/µl	0.5	0.5 U
DNA template		1	-
Nuclease-free water		19.5	
Total		25	

Table 2.9. Components of PCR for 3'-conserved segments

Reagent	Stock concentration	Volume (µl)	Final concentration
Forward primers	25 µM	0.5	0.5 µM
Reverse primers	25 µM	0.5	0.5 µM
dNTPs	10 mM	0.5	0.2 mM
10X *Taq* buffer	10 X	2.5	1 X
Taq DNA polymerase	1 U/µl	0.2	0.2 U
DNA template		1	-
Nuclease-free water		19.8	
Total		25	

Table 2.10. DNA sequence used in the real-time RT-PCR experiements

Gene		Sequence of primer / probe	Reference
rpoD	Forward	5'-GGGCTGTCTCGAATACGTTGA-3'	Quale *et al.*, 2006
	Reverse	5'-ACCTGCCGGAGGATATTTCC-3'	
	Probe	FAM-5'-TGCGGATGATGTCTTCCACCTGTTCC-3'-NFQ	
oprD	Forward	5′-CTACGGCTACGGCGAGGAT-3′	Quale *et al.*, 2006
	Reverse	5′-GACCGGACTGGACCACGTACT-3′	
	Probe	FAM-5'-CACCACGAAACCAACCTCGAAGCC-3'-NFQ	

Table 2.11. Components of real-time RT-PCR for *oprD* and *rpoD*

Reagent	Stock concentration	Volume (µl)	Final concentration
TaqMan EZ buffer	5	5	1 X
Manganese acetate	25 mM	3	3 mM
dATP	10 mM	0.75	0.3 mM
dCTP	10 mM	0.75	0.3 mM
dGTP	10 mM	0.75	0.3 mM
dUTP	20 mM	0.75	0.6 mM
Forward primer	100 µM	0.225	0.3 µM
Reverse primer	100 µM	0.225	0.3 µM
Probe	10 µM	0.625	0.2 µM
AmpErase UNG	1 U/µl	0.25	0.25 U
rTth DNA polymerase	2.5 U/µl	1	0.5 U
Total		25	

Table 2.12. Components of PCR for *mexT*

Reagent	Stock concentration	Volume (µl)	Final concentration
Forward primers	25 µM	0.5	0.5 µM
Reverse primers	25 µM	0.5	0.5 µM
dNTPs	10 mM	0.5	0.2 mM
10X *Taq* buffer	10X	2.5	1 X
Taq DNA polymerase	1 U/µl	0.2	0.2 U
DNA template		1	-
Nuclease-free water		19.55	
Total		25	

Table 2.13. Primers for *mexT*

Primer	Target	Oligonucleotide sequence	Product size (bp)	Reference
MexT F1	The first part of *mexT*	5′-AAAACCACCCGTCGTTATTG-3′	638	Quale *et al.*, 2006
MexT R1		5′-CAGTTCGTCGGTGTAGCTGA-3′		
MexT F2	The second part of *mexT*	5′-TCAGCTACACCGACGAACTG-3′	701	Quale *et al.*, 2006
MexT R2		5′-GGGATGACTGTTCCAT-3′		

Table 3.1. Prevalence of *P. aeruginosa* isolated from different patient specimens from January 2001 to May 2005 in the New Territories East Cluster hospitals

Specimen type	% (no.) of isolates in different specimens
Sputum	32.1% (45)
Bronchial and tracheal aspirate	30% (42)
Urine	19.3% (27)
Wound	12.1% (17)
[1]Others	6.4% (9)

[1]Others include body fluid, pus and miscellaneous

Table 3.2. Age and sex distribution of patients

	No. of isolate from		Total % (no.)
Age	Males	Females	
0 ~ 9	2	0	1.4% (2)
10 ~ 61	23	21	31.4% (44)
61 ~75	33	9	30% (42)
76 or above	40	12	37.1% (52)
Total	98	42	140

Table 3.3. Antimicrobial susceptibilities of imipenem-resistant *P. aeruginosa* isolated from patients in hospitals of the New Territories East Cluster from January 2001 to May 2005

Antimicrobial agents	Year of isolation	MIC (mg/L) Range	MIC_{50}	MIC_{90}	% of isolates resistant to breakpoint concentrations in mg/L in brackets
Aminoglyosides					
Amikacin					
	2001	8~32	16	16	7.7 (16)
	2002	4~>256	16	16	11.4
	2003	8~128	16	32	10.3
	2004	8~64	16	32	21.4
	2005	1~32	8	16	8
	Total	4~>256	16	32	11.8
Gentamicin					
	2001	1~8	4	8	31 (4)
	2002	0.25~>64	4	16	31.4
	2003	1~>64	4	16	28.2
	2004	≤0.06~64	4	32	11
	2005	0.12~8	4	8	12
	Total	≤0.06~>64	4	16	22.7
Netilmicin					
	2001	4~32	4	16	23 (8)
	2002	0.5~>128	8	32	25.7
	2003	4~128	8	16	20.5
	2004	2~>128	8	64	10.7
	2005	≤0.25~>128	4	16	12
	Total	≤0.25~>128	8	32	18.4
Tobramycin					
	2001	0.5>64	1	4	7.7 (4)
	2002	0.12~64	1	4	8.6
	2003	0.5~>64	1	>64	10.3
	2004	0.12~64	1	2	7
	2005	0.25~2	1	2	0
	Total	0.12~64	1	4	6.7
β-lactam					
Piperacillin					
	2001	4~512	32	256	15.4 (64)

Table 3.3 (Continued).

Antimicrobial agents	Year of isolation	MIC (mg/L) Range	MIC_{50}	MIC_{90}	% of isolates resistant to breakpoint concentrations in mg/L in brackets
Piperacillin	2002	2~256	32	256	25.7
	2003	4~512	32	256	25.6
	2004	4~512	32	512	28.6
	2005	≤2~1024	64	512	40
	Total	≤2~1024	32	256	27.1
Piperacillin/tazobactam					
	2001	4~256	64	256	15.4 (64)
	2002	4~512	32	256	20
	2003	4~512	32	256	20.5
	2004	4~256	32	64	25
	2005	4~256	64	256	36
	Total	4~256	32	256	23.4
Ticarcillin					
	2001	32~512	128	256	53.8 (64)
	2002	16~>1024	128	512	60
	2003	16~512	128	256	51.3
	2004	4~512	64	512	57.1
	2005	4~512	128	256	56
	Total	4~>1024	128	256	55.6
Ticarcillin/clavulanic acid					
	2001	16~512	64	256	46.2(64)
	2002	16~>1024	64	512	40
	2003	16~512	128	256	51.3
	2004	2~512	64	256	42.9
	2005	2~512	128	512	56
	Total	2~>1024	64	256	47.3
Cefoxitin					
	2001	>256	>256	>256	100 (16)
	2002	>256	>256	>256	100
	2003	16~>256	>256	>256	97.4
	2004	>256	>256	>256	100
	2005	>256	>256	>256	100
	Total	16~>256	>256	>256	99.5
Cefotaxime					
	2001	16~>128	>128	>128	100 (8)
	2002	8~>128	64	>128	97.1

Table 3.3 (Continued).

Antimicrobial agents	Year of isolation	MIC (mg/L) Range	MIC_{50}	MIC_{90}	% of isolates resistant to breakpoint concentrations in mg/L in brackets
Cefotaxime	2003	8~>128	128	>128	94.8
	2004	4~>128	128	>128	96.4
	2005	4~>128	128	>128	96
	Total	4~>128	128	>128	96.7
Ceftazidime					
	2001	0.5~128	32	64	61.5 (8)
	2002	1~128	4	128	31.4
	2003	1~128	8	64	48.7
	2004	0.5~>128	8	32	42.9
	2005	0.5~>128	8	128	44
	Total	0.5~>128	8	128	45.7
Ceftriaxone					
	2001	32~>128	>128	>128	100 (8)
	2002	16~>128	128	>128	100
	2003	8~>128	>128	>128	94.8
	2004	4~>128	>128	>128	96.4
	2005	4~>128	>128	>128	96
	Total	4~>128	>128	>128	97.4
Cefepime					
	2001	4~128	16	64	69.2 (8)
	2002	4~128	16	64	65.7
	2003	4~128	16	32	64.1
	2004	4~64	16	32	60.7
	2005	2~>128	16	64	80
	Total	2~>128	16	64	67.9
Aztreonam					
	2001	8~128	32	128	69.2 (8)
	2002	4~>128	16	128	60
	2003	4~>128	16	128	64.1
	2004	1~128	16	128	53.6
	2005	0.12~128	16	64	68
	Total	0.12~128	16	128	63
Imipenem					
	2001	8~32	16	32	100 (4)
	2002	8~128	16	32	100
	2003	8~64	16	32	100
	2004	8~32	8	16	100
	2005	8~32	8	16	100

Table 3.3 (Continued).

Antimicrobial agents	Year of isolation	MIC (mg/L) Range	MIC_{50}	MIC_{90}	% of isolates resistant to breakpoint concentrations in mg/L in brackets
Imipenem	Total	8~128	16	32	100
Meropenem					
	2001	4~128	16	>128	76.9 (4)
	2002	1~>128	16	>128	74.3
	2003	4~>128	16	>128	76.9
	2004	1~>128	8	64	75
	2005	1~>128	32	>128	88
	Total	1~>128	16	>128	78.2
Fluoroquinolones					
Levofloxacin					
	2001	0.25~8	2	8	7.7 (4)
	2002	≤0.12~64	1	4	5.7
	2003	0.25~64	1	8	15.4
	2004	0.25~>64	2	8	17.8
	2005	0.25~>64	1	8	20
	Total	≤0.12~>64	1	8	13.3
Norfloxacin					
	2001	0.5~8	1	8	15.4 (4)
	2002	0.5~>64	1	8	11.4
	2003	0.5~>64	1	8	12.8
	2004	0.5~>64	1	32	21.4
	2005	0.5~>64	1	32	16
	Total	0.5~>64	1	8	15.4
Ofloxacin					
	2001	0.5~32	1	16	30.8 (2)
	2002	0.25~>32	1	4	14.3
	2003	1~>32	2	16	30.8
	2004	0.25~>32	1	>32	32.1
	2005	0.5~>32	2	>32	32
	Total	0.25~>32	1	32	28

Table 3.4. Resistance patterns of imipenem resistant *P. aeruginosa* isolated from patients in hospitals of the New Territories East Cluster from January 2001 to May 2005

No. of antibiotic	Resistance pattern[1]	Total % (no.)
2	IMP CX	0.7 (1)
3	IMP CX CRO	1.4 (2)
4	IMP CTX CX CRO	14.3 (20)
5	IMP MEM CTX CX CRO	7.1 (10)
	IMP CTX PIP CX CRO	1.4 (2)
	IMP CX LVX NOR OFX	0.7 (1)
	Total (3)	9.3 (13)
6	IMP MEM AZM CTX CX CRO	2.1 (3)
	IMP MEM CTX CPM CX CRO	1.4 (2)
	IMP MEM CTX TIC CX CRO	0.7 (1)
	IMP CTX CPM CX CRO AN	2.1 (3)
	Total (4)	6.4 (9)
7	IMP MEM CAZ CTX CPM CX CRO	0.7 (1)
	IMP MEM AZM CTX CPM CX CRO	1.4 (2)
	IMP MEM CTX CPM TIC CX CRO	0.7 (2)
	IMP AZM CAZ CTX CPM CX CRO	1.4 (2)
	IMP CTX CPM CX CRO GM NET	1.4 (1)
	Total (5)	5.7 (8)
8	IMP MEM CTX CPM TIC T/C CX CRO	1.4 (2)
	IMP MEM CTX CPM CX CRO LVX OFX	0.7 (1)
	Total (2)	2.1 (3)
9	IMP MEM AZM CAZ CTX CPM PIP CX CRO	0.7 (1)
	IMP MEM AZM CAZ CTX CPM T/C CX CRO	1.4 (2)
	IMP MEM AZM CAZ CTX CPM TIC CX CRO	0.7 (1)
	IMP MEM AZM CTX T/C CX CRO NOR OFX	0.7 (1)

Table 3.4
(Continued)

No. of antibiotic	Resistance pattern[1]	Total % (no.)
	IMP MEM AZM CTX CPM TIC CX CRO OFX	1.4 (2)
	IMP CAZ CTX CPM CX CRO AN GM NET	0.7 (1)
	IMP AZM CAZ CTX CPM PIP CX CRO LVX	1.4 (2)
	Total (7)	7.1 (10)
10	IMP MEM AZM CAZ CTX CPM TIC T/C CX CRO	0.4 (1)
	IMP MEM AZM CAZ CTX CPM TIC PIP CX CRO	3.6 (5)
	IMP MEM AZM CAZ CTX TIC T/C PIP TZP CX CRO	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C CX CRO	0.7 (1)
	IMP MEM AZM CAZ CTX CPM CX CRO GM NET	1.4 (2)
	IMP MEM AZM CAZ CTX CPM CX CRO GM OFX	0.7 (1)
	IMP MEM AZM CTX CPM TIC T/C CX CRO OFX	3.6 (5)
	IMP MEM CTX CPM TIC T/C CX CRO GM NN	0.7 (1)
	IMP AZM CTX CPM TIC CX CRO AN GM NET	0.7 (1)
	Total (9)	12.9 (18)
11	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C CX CRO OFX	1.4 (2)
	IMP AZM CAZ CTX CPM TIC CX CRO GM NET OFX	0.7 (1)
	Total (3)	2.9 (4)
12	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO	5.7 (8)
	IMP MEM AZM CAZ CTX CPM TIC T/C CX CRO NOR OFX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C CX CRO LVX OFX	0.7 (1)
	IMP MEM AZM CTX CPM TIC T/C PIP TZP CX CRO OFX	1.4 (2)
	IMP MEM AZM CTX CPM TIC T/C CX AN NN NET NOR	0.7 (1)
	IMP MEM AZM CTX CPM TIC T/C CX CRO LVX NOR OFX	0.7 (1)
	IMP MEM AZM CTX CPM TIC T/C CX CRO GM NET OFX	0.7 (1)
	IMP MEM AZM CTX CPM CX CRO GM NN LVX NOR OFX	1.4 (2)
	Total (8)	12.1 (17)

Table 3.4 (Continued).

No. of antibiotic	Resistance pattern[1]	Total % (no.)
13	IMP MEM AZM CAZ CTX TIC T/C CX CRO GM NET NOR OFX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO OFX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CRO GM NET	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C CX CRO LVX NOR OFX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO GM	0.7 (1)
	IMP MEM AZM CTX TIC T/C CX CRO AN GM NN NET NOR	2.1 (3)
	IMP MEM AZM CTX CPM TIC T/C CX CRO AN LVX NOR OFX	0.7 (1)
	IMP MEM AZM CTX CPM TIC T/C CX CRO AN NET NOR OFX	0.7 (1)
	Total (8)	7.1 (10)
14	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO GM NET	2.1 (3)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO LVX OFX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO NOR OFX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM T/C PIP TZP CX CRO GM LVX OFX	1.4 (2)
	IMP MEM AZM CAZ CTX CPM TIC CX CRO GM NN NET LVX OFX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM PIP TZP CX CRO AN LVX NOR OFX	0.7 (1)
	IMP MEM AZM CTX CPM TIC T/C CX CRO GM NN LVX NOR OFX	1.4 (2)
	Total (7)	7.9 (11)
15	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO GM LVX OFX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO GM NET OFX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO LVX NOR OFX	1.4 (2)
	Total (3)	2.9 (4)
16	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO AN GM NET OFX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO GM NET NOR OFX	0.7 (1)
	Total (2)	1.4 (2)
17	IMP MEM AZM CAZ CTX CPM TIC T/C CX CRO AN GM NN NET LVX NOR OFX	1.4 (2)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO AN GM NET NOR OFX	0.7 (1)
	IMP MEM AZM CAZ CTX CPM TIC PIP TZP CX CRO AN GM NN NET NOR OFX	1.4 (2)

No. of antibiotic	Resistance pattern[1]	Total % (no.)
17	Total (3)	3.8 (5)
18	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO AN GM NET LVX NOR OFX	1.4 (2)
19	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO AN GM NN NET LVX NOR OFX	0.7 (1)
Total (71)		100 (140)

[1]IMP=imipenem MEM=meropenem AZM=aztreonam CAZ=ceftazidime CTX=cefotaxime CPM=cefepime TIC=ticarcillin T/C=ticarcillin/clavulanic acid PIP=piperacillin TZP=piperacillin/tazobactam CX=cefoxitin CRO=ceftriaxone AN=amikacin GM=gentamicin NN=tobramycin NET=netilmicin LVX=levofloxacin NOR=norfloxacin OFX=ofloxacin

Table 3.5. Results of phenotypic detection of MBL producing strains

		No. of strains with MICs of IMP[1] (mg/L)				
Increased zone diameters (mm)	Total no. of strains	8	16	32	64	128
2	1	1	0	0	0	0
3	12	7	4	1	0	0
4	33	16	16	1	0	0
5	35	16	15	3	1	0
6	25	6	17	8	0	0
7	14	3	7	4	0	0
8	8	2	1	3	1	0
9	4	0	1	2	0	1
10	3	0	1	2	0	0
11	1	0	0	1	0	0
12	4	0	3	0	1	0
Total:	140					

[1]IMP=imipenem

Table 3.6. The isoelectric points of β-lactamases produced by the isolates

Total no. of pI values	pI values	% of isolates (no.)
1	7.6	8.8 (8)
	7.7	8.8 (8)
	7.8	15.4 (14)
	7.9	11 (10)
	8.0	11 (10)
	8.1	9.9 (9)
	8.2	11 (10)
	Total (7)	75.8 (69)
2	7.6, 7.7	1.1 (1)
	7.6, 7.8	1.1 (1)
	7.7, 7.9	1.1 (1)
	7.9, 8.1	1.1 (1)
	8.0, 8.1	2.2 (2)
	Total (5)	6.6 (6)
3	7.6, 7.8, 8.0	1.1 (1)
	7.7, 7.9, 8.0	2.2 (2)
	7.7, 8.0, 8.1	1.1 (1)
	7.8, 8.0, 8.2	1.1 (1)
	7.9, 8.1, 8.2	2.2 (2)
	8.0, 8.1, 8.2	2.2 (2)
	Total (6)	9.9 (9)
4	7.6, 7.8, 8.0, 8.2	2.2 (2)
	7.7, 7.9, 8.0, 8.2	2.2 (2)
	7.8, 7.9, 8.0, 8.2	3.3 (3)
	Total (3)	7.7 (7)
	Total no. of pI patterns: 21	100 (91)

Table 3.8. Resistance patterns of *P. aeruginosa* harboring class 1 integron

No. of antibiotic	Resistance pattern[1]	Total % (no.)
2	IMP CX	1.1 (1)
3	IMP CX CRO	2 .1 (2)
4	IMP CTX CX CRO	17 (16)
5	IMP MEM CTX CX CRO	8.5 (8)
	IMP CTX PIP CX CRO	1.1 (1)
	IMP CX LVX NOR OFX	1.1 (1)
	Total (3)	10.6 (10)
6	IMP MEM AZM CTX CX CRO	2.1 (2)
	IMP MEM CTX CPM CX CRO	2.1 (2)
	IMP MEM CTX TIC CX CRO	1.1 (1)
	IMP CTX CPM CX CRO AN	2.1 (2)
	Total (4)	7.4 (7)
7	IMP MEM CAZ CTX CPM CX CRO	1.1 (1)
	IMP MEM AZM CTX CPM CX CRO	2.1 (2)
	IMP CTX CPM CX CRO GM NET	1.1 (1)
	Total (3)	4.2 (4)
8	IMP MEM CTX CPM TIC T/C CX CRO	1.1 (1)
	IMP MEM CTX CPM CX CRO LVX OFX	1.1 (1)
	Total (2)	3.2 (2)
9	IMP MEM AZM CTX CPM TIC CX CRO GM	1.1 (1)
	IMP MEM AZM CAZ CTX CPM PIP CX CRO	1.1 (1)
	IMP MEM AZM CAZ CTX CPM T/C CX CRO	1.1 (1)

No. of antibiotic	Resistance pattern[1]	Total % (no.)
9	IMP MEM AZM CAZ CTX CPM TIC CX CRO	1.1 (1)
	IMP MEM AZM CTX T/C CX CRO NOR OFX	1.1 (1)
	IMP CAZ CTX CPM CX CRO AN GM NET	1.1 (1)
	IMP AZM CAZ CTX CPM PIP CX CRO LVX	2.1 (2)
	Total (7)	8.5 (8)
10	IMP MEM AZM CTX CPM TIC T/C CX CRO OFX	4.3 (4)
	IMP MEM AZM CAZ CTX CPM TIC PIP CX CRO	3.2 (3)
	IMP MEM CTX CPM TIC T/C CX CRO GM NN	1.1 (1)
	IMP MEM AZM CAZ CTX TIC T/C PIP TZP CX CRO	1.1 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C CX CRO	1.1 (1)
	IMP AZM CTX CPM TIC CX CRO AN GM NET	1.1 (1)
	Total (6)	11.7 (11)
11	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX	1.1 (1)
12	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO	6 (6)
	IMP MEM AZM CAZ CTX CPM TIC T/C CX CRO LVX OFX	1 (1)
	IMP MEM AZM CTX CPM TIC T/C PIP TZP CX CRO OFX	1 (1)
	IMP MEM AZM CTX CPM TIC T/C CX AN NN NET NOR	1 (1)
	IMP MEM AZM CTX CPM TIC T/C CX CRO LVX NOR OFX	1 (1)
	Total (5)	10.6 (10)
13	IMP MEM AZM CAZ CTX TIC T/C CX CRO GM NET NOR OFX	1 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C TZP CX CRO GM NET	1 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO OFX	1 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C CX CRO LVX NOR OFX	1 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO GM	1 (1)

No. of antibiotic	Resistance pattern[1]	Total % (no.)
13	IMP MEM AZM CTX TIC T/C CX CRO AN GM NN NET NOR	2 (2)
	IMP MEM AZM CTX CPM TIC T/C CX CRO AN LVX NOR OFX	1 (1)
	IMP MEM AZM CTX CPM TIC T/C CX CRO AN NET NOR OFX	1 (1)
	Total (8)	9.6 (9)
14	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO GM NET	3 (3)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO LVX OFX	1 (1)
	IMP MEM AZM CAZ CTX CPM T/C PIP TZP CX CRO GM LVX OFX	1 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO NOR OFX	1 (1)
	IMP MEM AZM CAZ CTX CPM PIP TZP CX CRO AN LVX NOR OFX	1 (1)
	Total (5)	7.4 (7)
15	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO LVX NOR OFX	1 (1)
16	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO AN GM NET OFX	1 (1)
17	IMP MEM AZM CAZ CTX CPM TIC T/C CX CRO AN GM NN NET LVX NOR OFX	1 (1)
	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO AN GM NET NOR OFX	1 (1)
	IMP MEM AZM CAZ CTX CPM TIC PIP TZP CX CRO AN GM NN NET NOR OFX	1 (1)
	Total (3)	3.2 (3)
18	IMP MEM AZM CAZ CTX CPM TIC T/C PIP TZP CX CRO AN GM NET LVX NOR OFX	1 (1)
	Total (53)	100 (94)

[1]IMP=imipenem MEM=meropenem AZM=aztreonam CAZ=ceftazidime CTX=cefotaxime CPM=cefepime TIC=ticarcillin T/C=ticarcillin/clavulanic acid PIP=piperacillin TZP=piperacillin/tazobactam CX=cefoxitin CRO=ceftriaxone AN=amikacin GM=gentamicin NN=tobramycin NET=netilmicin LVX=levofloxacin NOR=norfloxacin OFX=ofloxacin

Table 3.9. Distribution of resistance genes in gene cassettes

Resistant gene	No of isolates with amplicon size in bp			Total % (no.)
	800	900	1000	
aadA1	3	0	0	4 (3)
aadA2	0	1	27	70 (28)
aadA6	1	3	3	9 (7)
bla_{OXA-3}	0	0	1	1 (1)
bla_{OXA-10}	0	0	1	1 (1)
Total:	4	4	32	85 (40)

Table 3.10. Susceptibility and SDS-PAGE of outer membrane proteins, and mRNA expression studies for the clinical isolates

Isolate no.	MIC (mg/L) of IMP[1]	MIC (mg/L) of MEM[2]	46-kDa protein present[3]	mRNA expression for oprD[4]	MexT amino acid substitution
2782	32	>128	No	0.09	Leu_{26}→Val Glu_{31}→Lys
543	16	>128	No	0.1	Leu_{26}→Val
2860[5]	8	2	No	0.1	Leu_{26}→Val Val_{110}→Phe Ser_{135}→Thr
1788[5]	32	>128	No	0.23	Leu_{26}→Val
1757	64	>128	No	0.27	Leu_{26}→Val Glu_{31}→Lys
1695	32	8	No	0.41	Leu_{26}→Val Glu_{31}→Lys
4782[5]	64	64	No	0.46	Leu_{26}→Val Glu_{31}→Lys
1287	16	16	Yes	0.49	Leu_{26}→Val
2389[5]	128	128	Yes	0.84	Not amplifiable
3767[5]	8	16	No	1.1	Leu_{26}→Val
939[5]	8	16	Yes	1.1	Leu_{26}→Val
455	16	>128	Yes	1.35	Leu_{26}→Val
2689[5]	32	>128	Yes	1.65	Leu_{26}→Val
52[5]	64	>128	Yes	10	Leu_{26}→Val
4346	16	2	Yes	11	Leu_{26}→Val
1127[5]	8	8	Yes	11	Leu_{26}→Val
1017[5]	8	32	Yes	11	Leu_{26}→Val

4397	8	16	Yes	2.12	$Leu_{26}\rightarrow Val$
1122	16	64	Yes	2.16	$Leu_{26}\rightarrow Val$
4247	8	8	Yes	3.3	$Leu_{26}\rightarrow Val$
88[5]	16	4	Yes	3.5	$Leu_{26}\rightarrow Val$
4639[5]	16	>128	Yes	4.1	$Leu_{26}\rightarrow Val$
2132[5]	8	8	No	4.9	$Leu_{26}\rightarrow Val$
360	16	>128	Yes	5.2	$Leu_{26}\rightarrow Val$
2015[5]	16	4	Yes	6.8	$Leu_{26}\rightarrow Val$
4225	16	16	Yes	8.4	$Leu_{26}\rightarrow Val$
2597[5]	16	4	Yes	9	$Leu_{26}\rightarrow Val$
4266[5]	32	>128	Yes	9.7	$Leu_{26}\rightarrow Val$
ATCC 27853	<4	<4	Yes	1	$Leu_{26}\rightarrow Val$

[1]IMP=imipenem

[2]MEM=meropenem

[3]Determined by SDS-PAGE of outer membrane proteins.

[4]Relative to the expression of *P. aeruginosa* ATCC 27853, which is assigned a value of 1.

[5]The second half of the *mexT* gene could not be amplified.

Appendix 1. Nucleotide sequence of *bla*OXA-10

Gen bank accession no. AM392427

ATGAAAACATTTGCCGCATATGTAATTATCGCGTGTCTTTCGAGTACGGCATTA
GCTGGTTCAATTACAGAAAATACGTCTTGGAACAAAGAGTTCTCTGCCGAAGC
CGTCAATGGTGTCTTCGTGCTTTGTAAAAGTAGCAGTAAATCCTGCGCTACCAA
TGACTTAGCTCGTGCATCAAAGGAATATCTTCCAGCATCAACATTTAAGATCCC
CAACGCAATTATCGGCCTAGAAACTGGTGTCATAAAGAATGAGCATCAGGTTT
TCAAATGGGACGGAAAGCCAAGAGCCATGAAGCAATGGGAAAGAGACTTGAC
CTTAAGAGGGGCAATACAAGTTTCAGCTGTTCCCGTATTTCAACAAATCGCCA
GAGAAGTTGGCGAAGTAAGAATGCAGAAATACCTTAAAAAATTTTCCTATGGC
AACCAGAATATCAGTGGTGGCATTGACAAATTCTGGTTGGAAGGCCAGCTTAG
AATTTCCGCAGTTAATCAAGTGGAGTTTCTAGAGTCTCTATATTTAAATAAATT
GTCAGCATCTAAAGAAAACCAGCTAATAGTAAAAGAGGCTTTGGTAACGGAGG
CGGCACCTGAATATCTAGTGCATTCAAAAACTGGTTTTTCTGGTGTGGGAACTG
AGTCAAATCCTGGTGTCGCATGGTGGGTTGGGTGGGTTGAGAAGGAGACAGAG
GTTTACTTTTTCGCCTTTAACATGGATATAGACAACGAAAGTAAGTTGCCGCTA
AGAAAATCCATTCCCACCAAAATCATGGAAAGTGAGGGCATCATTGGTGGCTA

Appendix 2. Nucleotide sequence of *bla*OXA-3

Gen bank accession no. L074945

ATTACTCGCTGCGCTCCTAATTTGCCGGTGAGCGTGGCGTTGGGCGTCAAGGA
AAACTTAATGGCAATCCGAATCTTTGCAATACTTTTCTCCACTTTTGTTTTTGGC
ACGTTCGCGCATGCACAAGAAGGCATGCGCGAACGTTCTGACTGGCGGAAGTT
TTTCAGCGAATTTCAAGCCAAAGGCACGATAGTTGTGGCAGACGAACGCCAAA
CAGATCGTGTCATATTGGTTTTTGATCAGGTGCGGTCAGAGAAACGCTACTCGC
CGGCCTCGACATTCAAGATTCCACATACACTTTTTGCACTTGACGCAGGCGCTG
CACGTGATGAGTTTCAAGTTTTCCGATGGGACGGCATCAAAAGAAGCTTTGCA
GCTCACAACCAAGACCAAGACTTGCGATCAGCAATGCGGAATTCTACTGTCTG
GATTTATGAGCTATTTGCAAAAGAGATCGGTGAAGACAAGGCTCGACGCTATT
TGAAGCAAATCGACTATGGCAACGCCGATCCTTCGACAAGTAATGGCGATTAC
TGGATAGATGGCAATCTTGCTATCGCGGCACAAGAACAGATTGCATTTCTCAG
GAAGCTCTATCATAACGAGTTGCCCTTTCGGGTAGAACATCAGCGCTTGGTCA
AGGACCTCATGATTGTGGAAGCCGGTCGCAACTGGATACTGCGCGCAAAGACG
GGCTGGGAAGGCCGCATTGGTTGGTGGGTAGGATGGGTTGAGTGGCCGACTGG
CCCCGTATTCTTCGCACTGAATATTGATACGCCAAACAGGATGGATGACCTTTT
CAAAAGGGAGGCAATAGTGCGGGCAATCCTTCGCTCTATCGAAGCGTTGCCGC
CCAACCCGGCAGTCAACTCGGACGCAGCGCGATAAAGCCGCGACAGC

Appendix 3. Nucleotide sequence of *Int*I1

Gen bank accession no. EF577407

CTACCTCTCACTAGTGAGGGGCGGCAGCGCATCAAGCGGTGAGCGCACTCCGG
CACCGCCAACTTTCAGCACATGCGTGTAAATCATCGTCGTAGAGACGTCGGAA
TGGCCGAGCAGATCCTGCACGGTTCGAATGTCGTAACCGCTGCGGAGCAAGGC
CGTCGCGAACGAGTGGCGGAGGGTGTGCGGTGTGGCGGGCTTCGTGATGCCTG
CTTGTTCTACGGCACGTTTGAAGGCGCGCTGAAAGGTCTGGTCATACATGTGAT
GGCGACGCACGACACCGCTCCGTGGATCGGTCGAATGCGTGTGCTGCGCAAAA
ACCCAGAACCACGGCCAGGAATGCCCGGCGCGCGGATACTTCCGCTCAAGGGC
GTCGGGAAGCGCAACGCCGCTGCGGCCCTCGGCCTGGTCCTTCAGCCACCATG
CCCGTGCACGCGACAGCTGCTCGCGCAGGCTGGGTGCCAAGCTCTCGGGTAAC
ATCAAGGCCCGATCCTTGGAGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGA
TCGAAATCCAGATCCTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCCC
GTTCCATACAGAAGCTGGGCGAACAAACGATGCTCGCCTTCCAGAAAACCGAG
GATGCGAACCACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGACGGCC
GAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCACCTTGCCG
TAGAAGAACAGCAAGGCCGCCAATGCCTGACGATGCGTGGAGACCGAAACCT
TGCGCTCGTTCGCCAGCCAGGACAGAAATGCCTCGACTTCGCTGCTGCCCAAG
GTTGCCGGGTGACGCACACCGTGGAAACGGATGAAGGCACGAACCCAGTGGA
CATAAGCCTGTTCGGTTCGTAAACTGTAATGCAAGTAGCGTATGCGCTCACGC
AACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCG
GTTTTCAT

Appendix 4. Nucleotide sequence of *aad*A1

Gen bank accession no. AF313472

ATGAGGGAAGCGGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGG
CGTCATCGAGCGCCATCTCGAACCGACGTTGCTGGCCGTACATTTGTACGGCTC
CGCAGTGGATGGCGGCCTGAAGCCACACAGTGATATTGATTTGCTGGTTACGG
TGACCGTAAGGCTTGATGAAACAACGCGGCGAGCTTTGATCAACGACCTTTTG
GAAACTTCGGCTTCCCCTGGAGAGAGCGAGATTCTCCGCGCTGTAGAAGTCAC
CATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACT
GCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAG
CCACGATCGACATTGATCTGGCTATCTTGCTGACAAAAGCAAGAGAACATAGC
GTTGCCTTGGTAGGTCCAGCGGCGGAGGAACTCTTTGATCCGGTTCCTGAACA
GGATCTATTTGAGGCGCTAAATGAAACCTTAACGCTATGGAACTCGCCGCCCG
ACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCGCATTTGGTACA
GCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAATG
GAGCGCCTGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACAGGCTTA
TCTTGGACAAGAAGAAGATCGCTTGGCCTCCCGCGCAGATCAGTTGGAAGAAT
TTGTTCACTACGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAA

Appendix 5. Nucleotide sequence of *aad*A2

Gen bank accession no. DQ091178

TGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACA
AAGTTAGACATCATGAGGGTAGCGGTGACCATCGAAATTTCGAACCAACTATC
AGAGGTGCTAAGCGTCATTGAGCGCCATCTGGAATCAACGTTGCTGGCCGTGC
ATTTGTACGGCTCCGCAGTGGATGGCGGCCTGAAGCCATACAGCGATATTGAT
TTGTTGGTTACTGTGGCCGTAAAGCTTGATGAAACGACGCGGCGAGCATTGCTC
AATGACCTTATGGAGGCTTCGGCTTTCCCTGGCGAGAGCGAGACGCTCCGCGC
TATAGAAGTCACCCTTGTCGTGCATGACGACATCATCCCGTGGCGTTATCCGGC
TAAGCGCGAGCTGCAATTTGGAGAATGGCAGCGCAATGACATTCTTGCGGGTA
TCTTCGAGCCAGCCATGATCGACATTGATCTAGCTATCCTGCTTACAAAAGCAA
GAGAACATAGCGTTGCCTTGGTAGGTCCGGCAGCGGAGGAATTCTTTGACCCG
GTTCCTGAACAGGATCTATTCGAGGCGCTGAGGGAAACCTTGAAGCTATGGAA
CTCGCAGCCCGACTGGGCCGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCC
GCATTTGGTACAGCGCAATAACCGGCAAAATCGCGCCGAAGGATGTCGCTGCC
GACTGGGCAATAAAACGCCTACCTGCCCAGTATCAGCCCGTCTTACTTGAAGC
TAAGCAAGCTTATCTGGGACAAAAAGAAGATCACTTGGCCTCACGCGCAGATC
ACTTGGAAGAATTTATTCGCTTTGTGAAAGGCGAGATCATCAAGTCAGTTGGTA
AATGATGTCTAACAATTCGTTCAAGCCGACCGCGCTACGCGCGGCGGCTTAAC
TCCGGCGTT

Appendix 6. Nucleotide sequence of *aad*A6

Gen bank accession no. DQ091179

TGATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACA
AAGTTAGACATCATGAGTAACGCAGTACCCGCCGAGATTTCGGTACAGCTATC
ACTGGCTCTCAACGCCATCGAGCGTCATCTGGAATCAACGTTGCTGGCCGTGC
ATTTGTACGGCTCTGCACTGGACGGTGGCCTGAAGCCATACAGTGATATTGATT
TGCTGGTTACTGTGGCTGCACGGCTCGATGAGACTGTCCGACAAGCCCTGGTC
GTAGATCTCTTGGAAATTTCTGCCTCCCCTGGCCAAAGTGAAGCTCTCCGCGCC
TTGGAAGTTACCATCGTCGTGCATGGTGATGTTGTCCCTTGGCGTTATCCGGCC
AGACGGGAACTGCAATTCGGGGAGTGGCAGCGTAAGGACATTCTTGCGGGCAT
CTTCGAGCCCGCCACAACCGATGTTGATCTGGCTATTCTGCTAACTAAAGTAAG
GCAGCATAGCCTTGCATTGGCAGGTTCGGCCGCAGAGGATTTCTTTAACCCAGT
TCCGGAAGGCGATCTATTCAAGGCATTGAGCGACACTCTGAAACTATGGAATT
CGCAGCCGGATTGGGAAGGCGATGAGCGGAATGTAGTGCTTACCTTGTCTCGC
ATTTGGTACAGCGCAGCAACCGGCAAGATCGCACCGAAGGATATCGTTGCCAA
CTGGGCAATGGAGCGTCTGCCAGATCAACATAAGCCCGTACTGCTTGAAGCCC
GGCAGGCTTATCTTGGACAAGGAGAAGATTGCTTGGCCTCACGCGCGGATCAG
TTGGCGGCGTTCGTTCACTTCGTGAAACATGAAGCCACTAAATTGCTTAGTGCC
ATGCCAGTGATGTCTAACAATTCATTCAAGCCGACGCCGCTTCGCGGCGCGGC
TTAATTCAGGCGTTAGTACCACTGAAACCCTCCTTTATTTCGCCCATGTTTATTC
AAACGGCATTCAGTTTCTCAAACGCTGTGCAGCGCTGGGTTTGCCGTTTCTCTG
GGCTTCGCCTGGTGGCGTTACGCTGGTTTGTGGTCTTTTTGGCCTCTGGCCCTTG
TGTAGCAAGCGCGAGCAGCTATTTTTTTCGTAGTGCTGTGCCGCCTCGGTGGCA
CCGTGCCTTTTCGCAGTTAGCGCCCGTCGCCAAGTTACGGTTATCCGTTTTGGC
TTCTGGCTCTAACATTTCGGTCAAGCCGACCCGCATTCTGCGGTCGGC
TTAACTCGCCCGTTAGATGCA

Appendix 7. Nucleotide sequence of *mex*T

Gen bank accession no. AJ007825.1

GGCCAGTTCGAAGCCGAGACCGGACGGCAGCGCAGCCTGCTCGGGGGCCAGG
TTCTGACGCCAGAGCACATCCTTCCAGCTCACGCCGATCGCCTGGACACGCATC
AGGACTTCCCCTGCGGCCGGCGCCGGGGTCGGCAGCTCTTCGCATTTGAGGAC
CTCTGGCGGGCCAAACTGATGAAAACGGATCACTCGGGACATCGCAAACCTCT
GCAGTGCATCACGGGGTGAATAACCTCATGGGTTGTGACTGTATCCGCCCATG
CCTGACAAAACCACCCGTCGTTATTGATAATGGCTATGCCTGTCAGTGATCCTA
TGCCCCTCCGGCACCTCGCCAGGCCCCGCCCCGTCTCGCACGCAAGGCTTGAC
GGCGAGCCCCCGCGGTTGCAGCCTCTAGCCCCTGGAAACGAGGAAACGCCATG
AACCGAAACGACCTGCGCCGCGTCGATCTGAACCTGCTGATCGTGTTCGAGAC
CCTGATGCACGAACGCAGCCTGACCCGCGCCGCAGAGAAACTGTTCCTCGGCC
AGCCGGCCATCAGCGCCGCGCTGTCGCGCCTGCGCACGCTGTTCGACGACCCG
CTGTTCGTCCGTACCGGACGCAGCATGGAGCCCACCGCGCGAGCCCAGGAAAT
CTTCGCCCACCTGTCGCCGGCGCTGGATTCCATCTCCACCGCCATGAGTCGCGC
CAGCGAGTTCGATCCGGCGACCAGCACCGCGGTGTTCCGCATCGGCCTTTCCG
ACGACGTCGAGTTCGGCCTGTTGCCGCCCCTGCTCCGCCGCCTGCGCGCGGAG
GCGCCGGGGATCGTCCTCGTCGTGCGCCGCGCCAACTATCTATTGATGCCGAA
CCTGCTGGCCTCGGGGGAGATCTCGGTGGGCGTCAGCTACACCGACGAACTGC
CGGCCAACGCCAAGCGCAAGACCGTGCGCCGCAGCAAGCCGAAGATCCTCCG
CGCCGACTCCGCGCCCGGCCAGCTGACCCTCGACGACTATTGCGCGCGACCGC
ACGCGCTGGTGTCCTTCGCCGGCGACCTCAGCGGCTTCGTCGACGAGGAGCTG
GAAAAATTCGGCCGCAAGCGCAAGGTGGTCCTGGCGGTGCCGCAGTTCAACGG
CCTCGGCACCCTCCTGGCCGGCACCGACATCATCGCCACCGTGCCCGACTACG
CCGCCCAGGCGCTGATCGCCGCCGGCGGCCTACGCGCCGAGGACCCACCGTTC
GAGACCCGGGCCTTCGAACTGTCGATGGCTTGGCGCGGCG
CCCAGGACAACGATCCGGCCGAACGCTGGCTGCGCTCGCGGATCAGCATGTTC
ATCGGCGATCCGGACAGTCTCTGAGCCCTCCGGCAGCTACCCGCACGAGGCGT
CGCAACGGGAAAATCGATCGCGCGCCGCGGGTGTGCGGCTTATTCCATCGAAA
GCACTGTCCATAACCATCGACTGTTACAGAAAACGAAAAACCAT
GTATCACTGTTCGTGAT

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